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Alternative Water Disinfection Schemes for Reduced Trihalomethane Formation. Volume 2 Algae as Precursors for Trihalomethanes in Chlorinated Drinking Water

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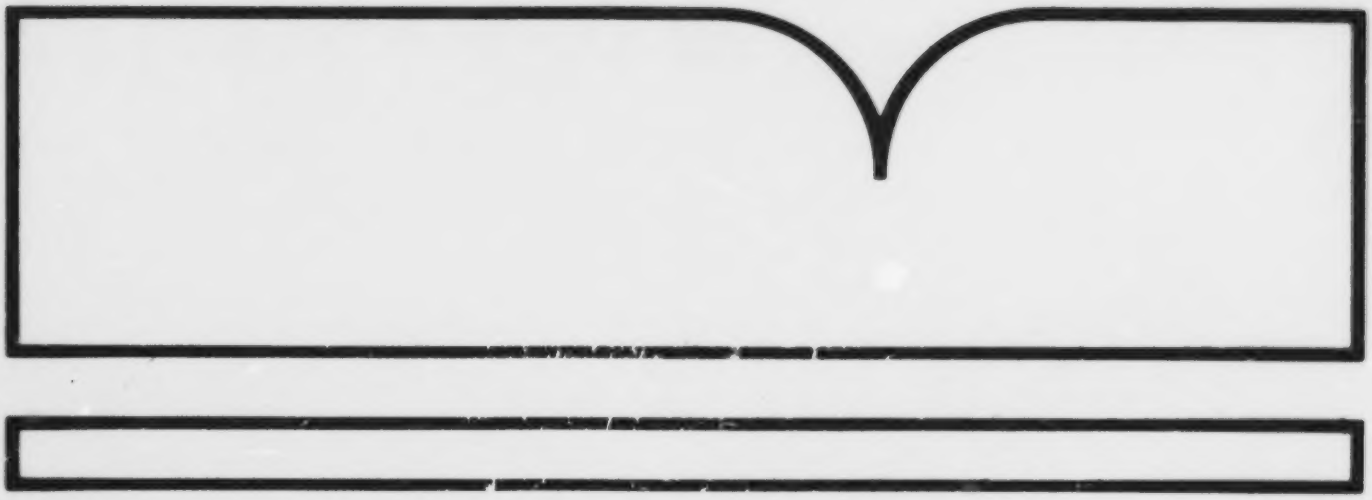

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ALTERNATIVE WATER DISINFECTION SCHEMES FOR REDUCED TRIHALOMETHANE FORMATION

Volume II. Algae as Precursors for Trihalomethanes in Chlorinated Drinking Water

by

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16. ABSTRACT

This study investigated three species of algae, Anabaena cylindrica, Scenedesmus quadricauda, and Pediastrum boryanum, with respect to their potential for the formation of THM when chlorinated. Algae were cultured and the cells (algal biomass) were separated from the extracellular products (ECP) at several times along the normal growth curves for each species in order to separately study their contribution as THM precursor material. The cells were resuspended in organic- and chlorine-demand free water and the cells and ECP were then separately dosed with chlorine at three chlorine concentrations. The THM formed after one hour and 24 hours of chlorine contact time were analyzed by the gas sparging technique and gas chromatography. For each time examined along the growth curve, monitoring of growth was done by cell counts and fluorometric assay of chlorophyll-a. Correlation of the growth period of the algae and THM production was observed. THMs were produced from both the ECP and the isolated algal cells of all three species when dosed with chlorine. The significance of these findings suggest that reduction of THM may be partially accomplished by observation and/or control of the natural phytoplankton communities in the water source for domestic water supplies.

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FORWORD

The U.S. Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimonies to the deterioration of our natural environment. The complexity of that environment and the interplay of its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution; it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems to prevent, treat, and manage wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, to preserve and treat public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research and provides a most vital communications link between the researcher and the user community.

This study was designed to evaluate trihalomethane production caused by chlorination of algal cells and algal extracellular products (ECP) separated from the cells. Both algal cells and algal ECP could form significant amounts of trihalomethanes. The findings have important implications for management of reservoirs that serve as sources for drinking water.

Francis T. Mayo, Director
Municipal Environmental Research
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ABSTRACT

Chlorination of water leads to the generation of trihalomethanes (THM). Algae as well as humic materials have been implicated as possible trihalomethane precursors. This study investigated Anabaena cylindrica, Scenedesmus quadricauda, and Pediastrum boryanum with respect to their potential for the formation of THM. Algae were cultured and the cells were separated from the extracellular products (ECP) at several times along the normal growth curves for each species. The cells were resuspended in organic- and chlorine demand-free water and the cells and ECP were then separately dosed with chlorine at three chlorine concentrations. The THM formed after one hour and 24 hours of chlorine contact time were analyzed by the gas sparging technique and gas chromatography. For each time examined along the growth curve, monitoring of growth was done by both cell counts and fluorometric assay of chlorophyll-a. Other chemical parameters determined for the samples included chlorine residuals (free and total), total organic carbon, total Kjeldahl nitrogen, ammonia-nitrogen, pH, and temperature. Correlation of the growth period of the algae and THM production was observed. Furthermore, significant levels of THM were produced from both the ECP and the isolated algal cells of all three species when dosed with chlorine. As expected, the THM levels formed were related to the free chlorine residual and to the TOC levels observed. The significance of these findings suggest that reduction of THM may be partially accomplished by observation and/or control of the natural phytoplankton communities in the water source for domestic water supplies.

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ABBREVIATIONS AND SYMBOLS

BOD	biochemical oxygen demand
C_a	concentration of air
$CHBr_3$	bromoform
CH_2Br_2Cl	dibromochloromethane
$CHBrCl_2$	bromodichloromethane
$CHCl_3$	chloroform
Cl_2	chlorine
cm	centimeter
cm^2	square centimeters
C_w	concentration of water
D	observed optical density
°C	degrees Centrigrade
ECP	extracellular products
EDTA	ethylene diamine tetracetic acid
g	gram
GAC	granular activated carbon
g/L	grams per liter
HOCl	hypochlorous acid
hrs	hours
ID	inside diameter
kg	kilogram
L	liter
LCV	leuco crystal violet
L/mole-cm	liters per mole-centimeter
mg/kg	milligrams per kilogram
mg/kg/day	milligrams per kilogram per day
mg/L	milligrams per liter
mL	milliliters
mL/L	milliters per liter
mm	millimeter
µg	microgram
µg/L	micrograms per liter
µL	microliter
µm	micrometer
N	normality
NCl_3	trichioramine
NH_2Cl	monochloramine
$NHCl_2$	dichloramine
NH_3-N	ammonia nitrogen
nm	nanometer
NVTOC	nonvolatile total organic carbon
OCl^-	hypochlorite ion
OD	outside diameter
PAC	powdered activated carbon
pH	hydrogen ion concentration
R^2	correlation coefficients
rpm	revolutions per minute
THM	trihalomethanes
TKN	total Kjeldahl nitrogen
TOC	total organic carbon
TTHM	total trihalomethanes
UV	ultraviolet
x g	times gravity

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SECTION 1

INTRODUCTION

It is widely accepted that the chlorination of some surface waters results in the formation of trihalomethanes. These compounds result from a reaction or series of reactions of chlorine with natural organic precursor material present in the source water. The exact nature of the precursor material is not clear, although humic and fulvic acids and algae have been implicated as contributors. Chloroform ($CHCl_3$) is the THM found in greatest quantities in finished waters but dibromochloromethane ($CHBr_2Cl$), bromodichloromethane ($CHBrCl_2$) and bromoform ($CHBr_3$) are commonly found in varying quantities, also. A maximum contaminant level of 0.10mg/L for trihalomethanes in finished waters has been set by the United States Environmental Protection Agency. Attempts to effectively reduce total trihalomethanes below the established levels in an economic way are hampered by the lack of a complete understanding of the nature of the THM precursors. Identification of THM precursors will allow establishment of techniques to selectively remove them.

The purpose of this study was primarily to answer some of the fundamental questions about the nature of trihalomethane precursors in surface waters. This information may aid in the development of alternate disinfection procedures allowing the reduction of finished water trihalomethane levels while still maintaining effective water disinfection.

SECTION 2

CONCLUSIONS

1. Significant levels of THM were produced from both the resuspended cells and the ECP at various stages of the growth curves of Anabaena, Pediastrum and Scenedesmus. The Anabaena cells provided as much or more THM as did the Anabaena ECP. The Scenedesmus ECP yielded more chloroform than did the corresponding algal biomass. For Pediastrum the chloroform provided by the ECP upon chlorination was comparable to the chloroform levels provided by the Pediastrum cells. The THM concentrations from all three algal species were comparable to yields of THM obtained in other studies evaluating humic and fulvic acids.

2. THM production from the Anabaena ECP increased as the stationary growth phase was approached. The THM production from the Anabaena cells also followed this trend.

3. Chloroform production from the Scenedesmus cells and ECP was initially somewhat high then declined until the late exponential/early stationary growth phase was reached, at which time maximal chloroform production occurred.

4. The Pediastrum cells and ECP provided two chloroform production maxima during the growth period examined. Both chloroform production maxima occurred during the period of growth considered to be the late exponential/early stationary growth phase.

5. THM production by the chlorination of Anabaena, Pediastrum and Scenedesmus cells and ECP was related to TOC level, to cell count (growth phase) and to chlorine residual after one hour of chlorine contact time.

6. The ECP chlorine residuals of all three organisms increased with culture age indicating that a change in type and/or degree of ECP produced was occurring. Alternately, a chlorine demanding component of the medium may have been assimilated by the cells.

7. Because cell count is related to THM production as well as to chlorophyll-a concentration along the growth curve, a relationship between chlorophyll-a and THM production for the algal cells is inferred. The ECP do not have significant levels of chlorophyll-a, indicating that because THM production occurs from both the cells and ECP, more than one THM precursor must be involved.

8. The isolated cells from all three organisms exhibited increased TOC, TKN, and NH_3-N levels with culture age. The ECP values of TOC, TKN, and NH_3-N for all three species were quite variable and the data probably indicates that the algae were producing different types and/or quantities of ECP along each species' growth curve.

SECTION 3

RECOMMENDATIONS

1. Coagulation and filtration of surface waters should precede chlorination in order to reduce THM levels in finished waters.

2. Algal blooming should be monitored and/or controlled so that water treatment procedures can be adopted that are appropriate to the source water conditions.

3. Further research recommended:

   (a) To identify which of the compounds that comprise algal ECP and cells are actually involved in THM production.

   (b) To investigate the role, if any, of algae-associated bacteria in THM production upon the chlorination of nonaxenic algae. For instance the THM production of axenic (bacteria-free) cultures of algae upon chlorination could be compared to the THM production of the same algae in nonaxenic culture in order to infer the role of the algae-associated bacteria in THM production.

   (c) To reproduce the experiments performed in this study to confirm the results and gain further information. Follow-up experiments should also measure 24 hour-chlorine residuals for the chlorinated ECP and cells to allow further analysis of THM data with respect to chlorine dose.

   (d) To generally investigate alternate disinfectant schemes such as chlorine followed by ammonia, ammonia followed by chlorine and chlorine dioxide, with respect to THM production from algae.

SECTION 4

LITERATURE REVIEW

BACKGROUND

There are many benefits from chlorination. Chlorine is a very effective and relatively inexpensive disinfectant. Chlorine is used as an oxidizing agent in water treatment plants for the removal of odors, reduction of biochemical oxygen demand (BOD), and as a disinfectant. Each milligram/L of chlorine reduces BOD by around 2 mg/L (Dugan, 1978). Dugan (1978) noted that the effectiveness of the chlorine depends on many factors, such as the amount and type of dissolved organic matter, temperature, pH, and the types of microorganisms to be destroyed. Also, the functional properties of chlorine as a disinfectant depend upon the amount of available chlorine in solution, called the residual chlorine.

Dugan (1978) has described the reactions involved in the chlorination process. Gaseous chlorine dissolves and reacts with water to form hypochlorous acid:

$$Cl_2 + HOH \rightleftharpoons HOCl + H^+ + Cl^-$$

The hypochlorous acid (HOCl) is a weak acid which can (depending upon the pH) dissociate when dissolved in water to form the hypochlorite ion:

$$HOCl \rightleftharpoons OCl^- + H^+$$

The HOCl and OCl^- have germicidal properties and together with Cl_2 are termed "free chlorine." The OCl^- has very weak germicidal properties compared to HOCl, however.

Chlorine reacts readily with many impurities in water and some of these reactions decrease the amount of chlorine available to inhibit microorganisms. Ammonium (NH_4^+) or ammonia (NH_3) react with chlorine to form chloramines. Chloramines have germicidal properties but they are much less effective than free chlorine (Hoehn et al., 1977b). The term "combined chlorine residual" refers to the chlorine that is in the chloramine form. The term "available chlorine" includes both the combined and free chlorine. Chlorine-consuming chemicals in water exert a "chlorine demand" that must be met before a free chlorine residual can be maintained in the water (Dugan, 1978).

Kleopfer and Fairless (1972) first discovered chlororganics in municipal drinking water from the Ohio River. This study demonstrated the presence of

chloroform, bromodichloromethane, dibromochloromethane and bromoform by the use of gas chromatographic and mass spectrometric techniques. Bellar et al. (1974) demonstrated by "unequivocal qualitative measurements" the presence of organochlorine compounds and some brominated hydrocarbons in tap water. The source water in this case was also the Ohio River and this raw water contained none or much lower concentrations of the organohalides than did the treated water. Finished water was found to contain trihalomethanes in concentrations as high as 35-150 µg/L. From these studies Bellar et al. (1974) proposed that a chemical reaction was taking place between free chlorine and organic compounds in the water, as opposed to these compounds being introduced as alum or chlorine impurities. Other more recent studies (U. S. Bureau of National Affairs, 1978) have detected chloroform at ppm levels as contaminants in chlorine. They concurred, however, that the principal source of trihalomethanes in drinking water is the chemical interaction of the chlorine added for disinfection with common natural organic precursors in the water.

Bellar et al. (1974) also correlated the organic content of the raw water with the halogenated product concentrations in finished water. For instance, water from sources that were low in total organics, such as well water, produced lower concentrations of halogenated products than surface waters that were naturally higher in total organics. Also, Symons et al. (1975) noted the correlation of organic content of water, as measured by the nonvolatile total organic carbon (NVTOC) test, to final trihalomethane concentrations when sufficient chlorine was added to create a chlorine residual. Furthermore, Rook (1976, 1977) clearly established a relationship between organic content of natural colored waters (fulvic and humic acids) and THM production.

Symons et al. (1975) conducted a study (National Organic Reconnaisance Survey) based on samplings from 80 locations of raw and finished water. Results of this study listed the median concentrations of trihalomethanes in finished water as: chloroform: 21 µg/L, with a range of 0.1 µg/L to 311 µg/L; bromodichloromethane: 6 µg/L, with a range of none found to 116 µg/L; and dibromochloromethane: 1.2 µg/L, with a range of none found to 100 µg/L. Bromoform was found in only 31.2 percent of the waters studied. The range for bromoform was none found to 92 µg/L. The four halogenated organics were either not present or were present only in low concentrations in the raw waters. Thus, it was concluded that the presence of trihalomethanes in finished water was due to chlorination practices. Dowty et al. (1975) also noted the increase in concentrations of many aromatic compounds, bromine-containing compounds and trihalomethanes by the water treatment of a municipal facility in the New Orleans area. However, the compounds found in the finished water were also found in the precursor river water, though generally in much lower quantities.

Coleman et al. (1977) used gas chromatographic/mass spectrometric techniques to characterize the volatile organics in finished drinking water in five cities: Miami, Florida; Seattle, Washington; Ottumwa, Iowa; Philadelphia, Pennsylvania; and Cincinnati, Ohio. This study found 72 volatile organic compounds in the finished waters, including chloroform, bromodichloromethane, dibromochloromethane, and bromoform. Halogenated organics accounted for 53% of the total organic compounds detected in the study and they accounted for

close to 50% of the number of compounds found in each city with the exception of Seattle (where halogenated organics made up only 30% of the compounds detected). Hoehn et al. (1977a) performed a study on the Occoquan Reservoir in Virginia, near Washington, D.C., in which chloroform was found to be the volatile organic appearing in highest concentrations with bromodichloromethane being the second most frequently observed volatile organic after standard chlorination was practiced.

Stevens and Symons (1977) noted that the reaction producing chloroform from chlorination disinfection is not an instantaneous reaction but that it occurs over a period of time until either the chlorine or the precursor is exhausted. The general reaction for the formation of chloroform and other trihalomethanes is given by:

$$\text{Chlorine + precursor (+ bromide ion and/or iodide ion)} \longrightarrow \text{chloroform (+ other trihalomethanes)}$$

In the presence of natural bromine, Stevens and Symons (1977) demonstrated the formation of bromodichloromethane, dibromochloromethane and bromoform. This occurred even when natural bromine was present only in small concentrations. Iodine containing THM species have also been observed due to the presence of natural iodide.

Bellar et al. (1974) rationalized the presence of bromodichloromethane and dibromochloromethane in municipal water supplies as most likely a result of bromine impurities contained in the chlorine used for disinfection. Bromoform was not present in levels above the detection limits of the procedures used in the study. The mixed chloro-, bromo-, and iodo- compounds may form by an initial oxidation of the halide ion in solution by the added chlorine, followed by rapid halogenation of organic precursors (Kleopfer, 1977). Bromination is preferential to chlorination of organic precursors in the presence of bromide ions due to the greater reactivity of bromine. Trussell and Umphres (1978) reported that the presence of bromide increases the yield of trihalomethanes for a given chlorine dose and may also increase the rate of trihalomethane development. The ratio of bromide to total organic carbon may be a factor in the final trihalomethane levels reached (Trussell and Umphres, 1978).

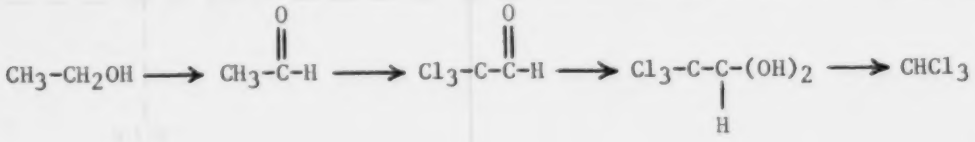
Kleopfer (1977) postulated that the oxidized form of the halogen undergoes the "haloform" reaction according to the following generalized reaction:

$$R\text{-}\overset{\overset{O}{\|}}{C}\text{-}CH_3 + 3OX^- \longrightarrow R\text{-}\overset{\overset{O}{\|}}{C}\text{-}O^- + CHX_3 + 2OH^-$$

Ten possible trihalomethanes could be produced in this manner, regardless of the precursors involved, if iodides and bromides are present in the water and if precursors that can undergo the haloform reaction are present. Only methyl ketones (or compounds that can be oxidized to this structural function) will undergo the haloform reaction (Figure 1). Morris and Baum (1977) also postulated that the major mechanism for production of trihalomethanes by chlorina-

tion of organic matter in water is the classical haloform reaction. The reaction was described as a base-catalyzed series of halogenation and hydrolysis reactions that typically occur with methyl ketones or compounds oxidizable to methylketones. Morris and Baum (1977) further theorized that in the formation of chloroform by the oxidation of organic carbon, sufficient chlorine must be present in order for complete oxidation of organic carbon to carbon dioxide and water to occur, except for the carbon that forms chloroform. Stevens et al. (1976) also suggested that more complex substances than methylketones could react by this mechanism or by some other mechanism that includes an oxidative cleavage step. Rook (1976, 1977) determined that a large number of other types of compounds react to produce THM products by mechanisms that must involve oxidative cleavage.

Bellar et al. (1974) postulated a possible haloform mechanism for the production of chloroform from ethanol. Ethanol oxidizes to acetaldehyde and the acetaldehyde reacts with free chlorine to form chloral. The chloral reacts with water to form chloral hydrate. Chloral hydrate then decomposes to form chloroform. This mechanism can be shown as:



No experimental evidence was provided by Bellar et al. (1974) to confirm this as the mechanism for chloroform production and they pointed out that there are virtually an infinite number of organic materials commonly found in waters that could react with free chlorine. Rook (1976) reemphasized this point and Stevens and Symons (1977) considered the chemical formation reactions of other trihalomethanes to be similar enough in mechanistic terms to discuss all these reactions as a group for general purposes.

Significant quantities of halogenated methanes do not result from the chlorination of ammonia-containing sewage (Stevens et al., 1976). Jolley (1975) and Glaze (1975) described other chlorinated organics that result from the chlorination of sewage effluents. Although trihalomethanes themselves do not result from chlorination of sewage, Glaze (1975) has shown evidence for three chlorinated acetone derivatives that are possible precursors of chloroform. Hoehn et al. (1977a) found that discharges of chlorinated sewage to a receiving tributary near Washington, D.C. did not appreciably alter the trihalomethane concentration. The techniques of Bellar and Lichtenberg (1974) enabled Hoehn to detect trihalomethane levels as low as 0.5-1 μg/L. Even though very low levels were measurable, the trihalomethane concentration changes were not found to be significant. It was postulated that either these effluents contained little chloroform or that some natural process, such as stream turbulence, caused its removal.

The primary reason for public concern about the formation of trihalomethanes in drinking water supplies is that these compounds may be carcinogenic to humans or hazardous to health in other ways. Page and Harris (1976) have

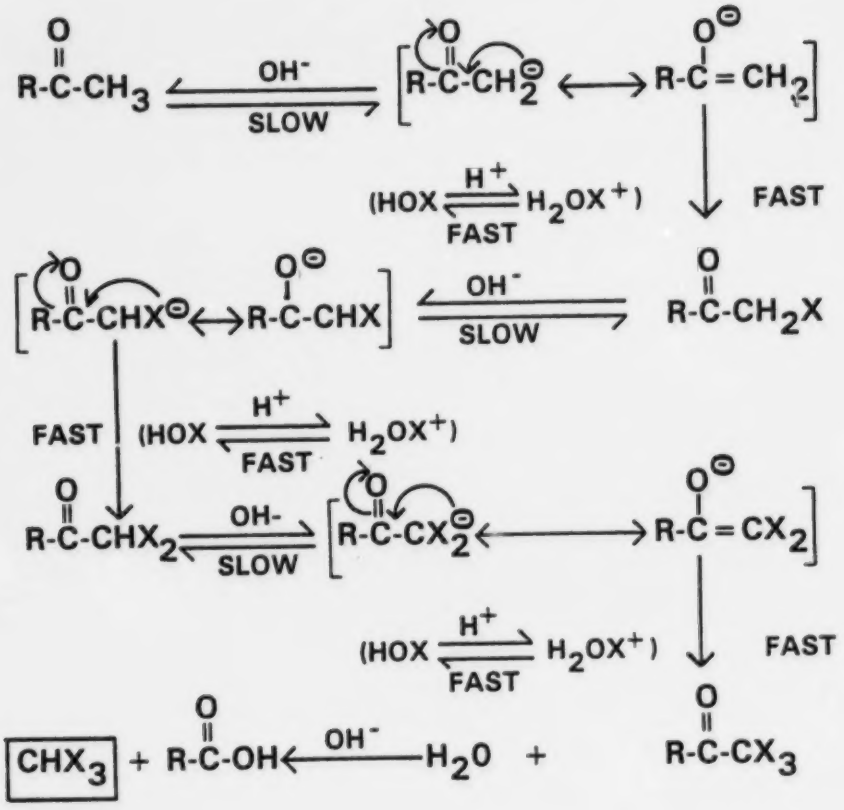


FIGURE 1. The Haloform Reaction (Morris and Baum, 1977)

supported the hypothesis that there is a link between carcinogens in drinking water and cancer mortality. The health dangers of trihalomethane formation from chlorination disinfection must be weighed against the dangers of altering a method of disinfection that has been proven effective. Hoehn et al. (1977b) have discussed the paradox of trying to protect public health by chlorination when this practice increases the concentrations of volatile halogenated organics, including trihalomethanes, in many potable waters.

The basic criteria for establishing chloroform as a carcinogen have been met (Tardiff, 1977). These criteria include statistically significant increases in neoplasia in treated animals over the spontaneous incidence in control animals, a dose-related increase in the incidence of cancer, time-to-tumor incidence data demonstrating a decrease in latency with increasing dose, and reproducibility not only within the same strain and species but also within other species. Tardiff (1977) noted that chloroform has produced tumors in more than one species (mouse and rat) and in more than one strain of mouse. Furthermore, a dose-response relationship of malignancies and other effects in mammals is evidence that these effects are compound related. In an effort to extrapolate findings of animal carcinogenicity to man, Tardiff (1977) stated that, quantitatively, man and mouse appear to metabolize chloroform by similar pathways. Available epidemiological data cited showed that 23-27 mg/kg/day for a 70 kg person over a 10 year year exposure period produced only reversible hepatotoxicity. Lower doses of 0.34-0.96 mg/kg daily over a 1-5 year period did not produce hepatotoxicity based on liver function. It was concluded that hepatotoxicity in humans may require oral doses of greater than 1 mg/kg/day but less than 25 mg/kg/day.

At present there is no direct evidence of chloroform carcinogenicity in humans but on the basis of rodent data, the likely organs for chloroform to affect most in man would be the kidney and liver. Tardiff (1977) considered that if drinking water doses are 0.01 mg/kg/day, chloroform in water may be responsible for as much as 1.6 percent of the current yearly human liver cancer incidence in the United States and for as much as 1.44 percent of the the yearly kidney cancer. Consequently, Tardiff recommended that the maximum chloroform concentration in tap water should be less than 70 µg/L to ensure a dose of less than 0.01 mg/kg/day. This level was defined as the recommended maximum concentration for total haloform based on infant protection and safe infant intake levels assuming that a 7-10 kg infant may consume one liter of water per day if a powdered milk formula is used. A maximum contaminant level of 0.10mg/L total trihalomethanes has been set by the United States Environmental Protection Agency for systems serving populations of greater than 10,000 persons.

Morris and Baum (1977) have pointed out that health risk assessments should be based on the total number of carbon-chlorine bonds rather than on the extent of chloroform or haloform formation only. This was suggested because the most simple chlorinated compounds (chloroform intermediates) and some non-volatile halogenated organics formed by chlorination also show possible carcinogenic risks.

TRIHALOMETHANE PRECURSORS

The precise substances and compounds that serve as trihalomethane precursors in natural waters are still a mystery. It is likely that several organic materials in surface waters serve as these precursors. Rook (1976) inferred that the precursors for the haloform reaction should be substances of extremely low vapor pressure and compounds rather resistant to biodegradation. If the precursor substances were not of low vapor pressure they would evaporate before reaction with halogens could occur. The large molecules of the humic and yellow fulvic acids were concluded by Rook to match these requirements. Additional studies by Rook (1977) and Morris and Baum (1977) have indicated that a wide variety of organic substances found in natural waters may contribute to the production of chloroform.

An aromatic ring with two hydroxyl (-OH) groups in the meta position as well as cyclohexane rings with the $-C(=O)-CH_2-C(=O)-$ configuration (for instance indandione and dimedon) are well suited for oxidative cleavage. Furthermore, Rook (1976) demonstrated that at pH 11 the reaction rates were increased compared to those rates at lower pH values. Therefore, he postulated that the chlorine reaction requires a reaction analgous to the enolization of aliphatic ketones. The ionization of phenolic OH into the phenoxide ion in the case of hydroxybenzenes is a pH dependent system (Rook, 1976).

Fulvic acid that was freshly extracted from peat obtained a range of haloform yields of 0.3-0.9%, depending on the chlorination conditions (Rook, 1976). Low concentrations of haloforms formed from the fulvic substances were explained on the basis of only a small number of meta dihydroxy structures present in the fulvic acid molecule. An alternate explanation for the low yields may be that there is chlorine substitution on the ring structures which would yield nonvolatile chlorinated products. Chlorine substitution at the few nitrogen atom sites present in peptide or other amino structures in fulvic acids is also likely.

Stevens et al. (1976) tested Rook's hypothesis of humic acids as potential trihalomethane precursors upon chlorination. These researchers showed that the rate curve for formation of trihalomethanes by chlorination of commercially obtained humic acid in concentrations similar to those found in the Ohio River (around 3 mg/L of nonvolatile total organic carbon) was very similar to the rate curve seen for chlorination of natural Ohio River water. The physical and chemical characteristics in terms of rate of trihalomethane formation upon chlorination of humic acid in suspension at the solution concentration used by Stevens et al. (1976) were found to be similar at pH 7 to those characteristics of the unknown precursor substances in the Ohio River. Experiments by Stevens et al. (1976) also illustrated a first order rate dependence of total trihalomethane reaction rate curves on initial humic acid concentration.

Hoehn et al. (1976b) have also indicated that humic substances common to surface waters are products of plant decomposition and are comprised principally of humic and fulvic acids. These compounds are complex, high molecular weight constituents of soils and decomposing vegetation. Under alkaline

conditions the humic acids were extracted from soils and then precipitated from solution by acidification. Fulvic acids remained in solution (Hoehn et al., 1977b). The oxygen-containing groups such as carboxyl groups, hydroxyl groups and alcoholic hydroxyl groups play an important role which make humic acids one of the principal reactants in the haloform reaction occurring when humic-containing waters are chlorinated (Hoehn et al., 1977b). No two molecules of humic acid are exactly the same so the reaction pathways for chloroform and other trihalomethanes from their humic acid precursors are difficult to identify because of the uncertainty of the exact structures involved (Rook, 1977).

Fulvic acids make up the major portion of organic matter in unpolluted colored surface or ground waters (Rook, 1977). Fulvic acids are a subgroup of yellow acids within humic acids that give waters a yellow color. These yellow acids are small size humic acids that remain in solution at pH 1. Rook (1977) has indicated that the most readily reacting site of the fulvic acid molecules for the haloform reaction is the carbon between two meta hydroxyl groups. The studies indicated that the treatment of fulvic acids in aqueous solution with halogens in neutral or alkaline conditions is "essentially an oxidative halogenating degradation method which effects dramatic alterations by simultaneous oxidations and substitutions." (Rook, 1977). This results in a number of fulvic acid degradation products. Chloroform may be a major product or it may be a by-product of side reactions. The pathway for trihalomethane production from fulvic acids as proposed by Rook involves a fast chlorination of carbon atoms that are activated by ortho hydroxyl substituents, or phenoxide ions in an alkaline environment. This leads to chloroform or mixed bromochlorohaloforms and/or iodination in the presence of bromine and/or iodine. Babcock and Singer (1979) have shown that fulvic acid may yield less chloroform and consume less chlorine than an equivalent amount of humic acid. Their study also noted that when insufficient chlorine is applied, all of it is consumed, chloroform production ceases after only a few hours and that larger amounts of other chlorinated organics are probably formed. Oliver and Lawrence (1979) found, however, that humic and fulvic acids at equal concentrations yield approximately the same amounts of chloroform.

Chol and Chen (1976) found that the total organic carbon level of sediments, as well as fulvic and humic acid fractions, bear a linear relationship with respect to concentrations of chlorinated hydrocarbons in sediments. The suspended humic particles may serve to transport chlorinated hydrocarbons through the water column and thus may provide a mechanism for accumulation of chlorinated hydrocarbons in sediments (Chol and Chen, 1976). These hydrocarbons could possibly be released by the processes involved in routine water treatment.

Stevens et al. (1976) observed that the rate of formation of chloroform is pH dependent with the rate of formation increasing as pH is increased from pH 6.5 to 11.5. They postulated that this pH dependency of rate on formation is explained by compounds in the source water that do not react readily at pH 6.5 but become significant contributors to the overall reaction at pH 11. Acetone was cited as one possible compound that could react in a classical haloform manner. However, because the rate of total trihalomethane production

is proportional to the acetone concentration, in order for acetone to be a significant precursor at pH > 6.5 much more acetone would have to be found in solution than is commonly present in natural waters. Additional data demonstrated that the characteristic rate curve for the haloform reaction of acetone to chloroform did not match the observed rate of trihalomethane production. Morris and Baum (1977) also doubted that acetone is a significant trihalomethane precursor under usual chlorination conditions because acetone and simple methyl ketones react too slowly to explain the formation of chloroform in the concentrations observed. For these reasons it is unlikely that acetone is a significant precursor for trihalomethanes at near neutral pH. For pH values much higher than 6.5, Stevens et al. (1976) showed that the rate of trihalomethane production from acetone was much faster than the trihalomethane production from of humic acid. They concluded that low molecular weight compounds containing the acetyl moiety that have reaction rates comparable to acetone could be significant precursors for trihalomethane production when high pH chlorination conditions prevail. Trihalomethane precursors are probably mixtures of compounds that react differently at varying pH values and their relative importance to trihalomethane production is dependent upon pH in a complex way.

Algal metabolites (extracellular organic products) and/or algal vegetative cells, both in living and decomposed states, may also serve as trihalomethane precursors. Hoehn et al. (1977b) noted a definite correlation between chlorophyll-a concentrations and total trihalomethane concentrations in a reservoir near Washington, D.C. Since it was known that the chlorophyll content of an algal group varies with the age of the algal population (Fruh et al., 1966), it was possible to establish a relationship between algal growth in the reservoir and concentrations of total trihalomethanes in the finished waters. This study provided the first indication that algae may be important as trihalomethane precursors.

Larson and Rockwell (1979) showed that aquatic microflora could serve as trihalomethane precursors upon dosing with chlorine. They theorized that aquatic microflora metabolic activity may be the principal source of citric acid in natural waters. Their work indicated that chloroform is produced from several naturally occurring carboxylic acids when treated with a tenfold mole excess of hypochlorite solution. Three carboxylic acids related to resorcinol gave good yields of chloroform. Citric acid and 3-ketoglutaric acid (produced in nature by enzymatic oxidative decarboxylation of citric acid) also gave quantitative yields of chloroform. In their study, microorganisms (a mixed population) were cultured and the supernatant of disrupted cells was partioned by ion exchange chromatography into a strongly acidic fraction (containing citric acid and other polycarboxylic acids) and a less acidic fraction. The two fractions were chlorinated separately and chloroform was produced only from the strongly acidic fraction. Larson and Rockwell (1979) also added that humic and fulvic acids may form chloroform via enzymatic degradation of lignin and related polyphenols which are possible precursors of 3-ketoglutaric and citric acid in treated and natural waters.

Compounds which ionize fairly rapidly to form carbanions are acceptable precursors for haloform production (Morris and Baum, 1977). β-ketones and

pyrrole rings are examples of such compounds. Their study demonstrated that hydrogens ortho to the nitrogen in pyrrole rings may be activated like those in phenol and, thus, provide possible sites for chlorination and subsequent haloform formation. Examples of such pyrrole ring compounds are chlorophylls, xanthophylls, heme, indole and its derivatives (like tryptophan). Related to the β-ketonic structures are acetogenins which are compounds that include many natural pigments. The reactions of carbonyls, especially those of the -diketones often resemble those of ketones. Phloroacetophenone and orsellinic acid are representatives of the acetogenin group of compounds. Many of these compound types are found in the extracellular metabolites produced by algae.

The green chlorophylls, chlorophyll-a and chlorophyll-b, and the red carotenoid pigments, xanthophylls, are lipid soluble pigments that are found in algae within the cytoplasm of the protoplasm (Weier et al., 1970). All divisions of algae contain chlorophyll-a and there may be accessory pigments associated with chlorophyll-a such as other chlorophylls, phycobilins, and carotenoid pigments (Alexopoulos and Bold, 1971). Carotenoid pigments are carotenes (yellow) and xanthophylls (red). Carotenes are composed of oxygen-free, long chain, unsaturated hydrocarbons, while xanthophylls are oxygenated derivatives of carotenoids (Alexopoulos and Bold, 1971). An example of a carotene that is commonly distributed in algae is β-carotene. Fucoxanthin and zeaxanthin are commonly found xanthophylls (Alexopoulos and Bold, 1971).

Morris and Baum (1977) showed that "soluble" chlorophyll yields chloroform when chlorinated with aqueous chlorine and the concentration produced is related to the pH of the solution. The yields of chloroform from the chlorinated chlorophyll increased as the pH value of the solution was increased from 5.8 to 10. Since chlorophyll, even if only at elevated pH values, can yield substantial amounts of chloroform, therefore algae as well as fulvates may be precursors of haloforms during water supply treatment. Hoehn et al. (1977b) concurred that humate sources of trihalomethane precursors may have algal precursors superimposed upon them in the spring and summer.

Echelberger et al. (1971) studied the effect of chlorine on algae and found that chlorination resulted in a reduction of cellular chlorophyll fractions. The presence of a chlorine residual can lead to cell lysis or cellular metabolite leakages. Because the cellular fractions of chlorophyll-a and -b are reduced, a release of organic matter measured as chemical oxygen demand into the suspending water may result. This increase in chemical oxygen demand of the water will increase the oxygen or chlorine demand so that, if sufficient times are allowed, a portion of this oxygen-demanding material will be oxidized by the residual chlorine. This can result in the formation of chlorinated organic compounds possibly including trihalomethanes themselves. The algal metabolites released by chlorination are described as high molecular weight polymeric materials, and these materials can cause flocculation of the algae because they can serve as structures for the algal suspension, through polymeric bridging from the algal cells, into three dimensional floccules of a large enough size to settle out of solution (Echelberger et al., 1971).

Hoehn et al. (1979) examined algae (algal cells and extracellular products) as precursors of trihalomethanes upon chlorination. Two green algae (Chlorella pyrenoidosa and Scenedesmus quandricauda) and two blue-green algae (Oscillatoria tenius and Anabaena flosaquae) were cultured. The extracellular products were separated from the cells of each species. Each species' cells (resuspended in distilled water) and extracellular products (ECP) were dosed with chlorine separately. From their experiment it was concluded that all four algal species produced ECP which, upon chlorination, yielded as much chloroform per unit organic carbon as that reported from humic and fulvic acid. The algal ECP produced greater yields of chloroform from available total organic carbon than did the corresponding algal biomass. Hoehn et al. (1979) also noted that there were indications that algae in the exponential phase of growth yielded higher amounts of trihalomethane precursors than during other phases of their life cycle. They concluded that this was probably due to a lack of sufficient chlorine added to meet the chlorine demand of the later growth stages. However, Hoehn and coworkers further noted that it was possible that different extracellular metabolites with lower trihalomethane yielding capacities were present during the later growth phases.

One of the methods of measuring biological productivity and the eutrophication level of waters is to determine the chlorophyll content of surface waters at intervals over a growing period (Sawyer, 1966). Oligotrophic water bodies have chlorophyll concentrations of up to 5 μg/L while eutrophic waters tend to have chlorophyll concentrations of greater than 6 μg/L (Jones et al., 1979). The volume of algae (or algal cell counts) in a body of water can also be used as a measurement of eutrophication (Sawyer, 1966; Fruh et al., 1966; Oswald and Goloeke, 1966). The nature of the organisms in a lake or reservoir may also allow determination of the degree of eutrophication, assuming that certain organisms are indicators of the onset of eutrophication (Sawyer, 1966). Fruh et al. (1966) stated that the measurement of the abundance, the rates of production, and the types of biological communities in relation to the lakes nutrient flux should give a reliable index of eutrophication. Because chlorophyll-a levels, algae, and eutrophication of a body of water are all interrelated and because chlorophyll may be a precursor for the formation of trihalomethanes by chlorination of surface waters, the level of eutrophication of a body of water used as supply for potable water may be of concern in the reduction of trihalomethanes. Jones and Bachmann (1976) have discussed a method of predicting the chlorophyll levels in lakes.

FACTORS AFFECTING TRIHALOMETHANE LEVELS

The organic content of the source water, temperature, season of year, pH, and chlorine dose and contact time have also been shown to influence the concentration levels of trihalomethanes in finished waters. Symons et al. (1975) noted that the dominant factor influencing the creation of chlorination by-products was the organic level of the source water, provided that there was a chlorine residual at the time of sampling. These workers argued that since the majority of the waters studied contained chlorine residuals the concentration of the end product (total trihalomethanes) was related to the concentration of the other reactants (unknown organic precursors). Consequently, measurement of the nonvolatile total organic carbon concentration of waters is

a reasonable indication of the concentration of these unknown precursors. Babcock and Singer (1979) found that in humic acid solutions in which excess chlorine was available, there was a linear relationship between amount of total organic carbon (TOC) present and chloroform produced. Young and Singer (1979) also found that the total organic carbon concentrations markedly influenced the formation of chloroform in finished waters. Because surface waters normally contain more organic matter than groundwaters, higher total trihalomethane concentrations are generally produced from surface water than from well water upon chlorination (Bellar et al., 1974 and Symons et al., 1975).

Raw water chlorination (prechlorination practice) tends to result in higher concentrations of total trihalomethanes than that resulting from only post chlorination (Symons et al., 1975), presumably due to the fact that the content of organic matter is greater in raw water than it is after coagulation, settling and filtration. Concentrations of trihalomethanes in finished waters can be reduced by chlorinating at the point in the treatment process where organic precursor content of the water is lowest. Delaying chlorination until after coagulation and clarification can significantly reduce the trihalomethane formation potential (Hoehn et al., 1977b).

There is a general relationship between quantities of trihalomethanes produced and water temperature. Concentrations of trihalomethanes in finished water for a given water source tend to increase as water temperature increases. Concentrations of trihalomethanes in finished waters at a given location also tend to increase in summer months, though this may be due to the temperature increases that accompany seasonal changes. Stevens et al. (1976) proposed that temperature differences (around 26°C) could account for most of the observed winter-to-summer changes in chloroform concentrations detected in their study (<30 µg/L to >200 µg/L). These researchers reported that almost twice as much chloroform was formed in a given time period by chlorinating natural water at 26°C as was formed at 2°C. This range is close to that of the United States seasonal temperature variations of surface waters. Hoehn et al. (1977a) also found that the formation of trihalomethanes was influenced by temperature. Regression analysis between observed temperatures and total trihalomethane concentrations showed a good correlation. However, this relationship was coincidental, since in this system the controlling parameter was the improved water quality of the reservoir studied, as reflected by lower chlorine demand in the winter. They also observed that high variations in weekly analyses of total trihalomethane concentrations occurred, with concentrations lower in the late fall and early winter than in the summer months. Hoehn et al. (1977a) suggested that although the concentrations of organics may fluctuate only a few milligrams per liter from winter to summer, the structure of the organics themselves could vary enough so that very different volatile organic concentrations would occur upon chlorination of these waters. Thus, these seasonal total trihalomethane concentration variations may be due to rainfall intensity changes in the watershed, changes in the degree of reservoir mixing with the season, in addition to temperature variations.

Under conditions of high pH the concentrations of trihalomethanes increase and the rates of formation increase when waters are chlorinated. Rook

(1976) showed that when pH values were increased from 8 to 10 a large reaction rate increase of formation of haloforms occurred from fulvic acids. This was explained as an increase in the formation of phenoxide ions with the increase in pH. The influence of pH changes within one unit of pH 7 were not significant. Stevens et al. (1976) postulated that final trihalomethane product concentrations increase when chlorination is performed at higher pH due to the presence of certain reactive sites on the complex humic acid molecule that do not react at significant rates at lower pH values. Rook (1977) noted that chlorination of single uncomplicated polyhydroxybenzenes involved various competing reactions. Reaction conditions such as the excess of one of the reactants or altered pH will determine which type of reaction prevails. Minor amounts of chloroform might be generated by secondary pathways that are favored by increased pH. Furthermore, Morris and Baum (1977) found that yields of chloroform could be increased when mixtures were made alkaline an hour or two before analysis for chloroform. This was true for proline, uracil, tryptophane and O-vanillin. These increases in chloroform yields were not accompanied by significant changes in chlorine consumption (demand) and in some cases yields were shown to increase in this manner even when solutions were dechlorinated prior to the pH change. These researchers concluded that this was indicative of the presence of intermediate chlorinated compounds, like trichloroacetate, that require alkaline conditions for hydrolysis.

Chlorination of surface waters at higher pH values were shown by Stevens and Symons (1977) to yield higher trihalomethane formation potentials. These researchers warned that if the pH used for laboratory studies is of a higher value than the pH for the same water measured in the distribution system then such reactions at a higher pH may involve different portions of the total precursors than those involved in the distribution system. Consequently, the reactions at higher pH may then be of no importance to the consumer since THM formation would not be equivalent in the two cases.

Chlorine dose and contact time have been shown to influence the trihalomethane concentrations in finished waters. Symons et al. (1975) observed that higher chlorine residuals in the finished water tended to give higher total trihalomethane concentrations and that free chlorine residuals of greater than 0.4 mg/L enhanced the formation of trihalomethanes. Hoehn et al. (1977b) indicated that the maximum trihalomethane production expected during normal water treatment processes (given adequate time for completion of the reactions) is a function of the amount of chlorine employed. Bellar et al. (1974) found that each time additional chlorine was added to increase or maintain the free chlorine concentration of the water, the chloroform levels also increased significantly. Moore et al. (1979) also found that the chlorine dose was a major factor influencing variations in the chloroform levels of 19 chlorinated water supplies. Chloroform concentration would be expected to be correlated to chlorine dose if organics in surface waters are chloroform precursors because as organic content of the waters increases, more chlorine must be added to produce an acceptable chlorine residual. Increasing the chlorine contact time, such as storage of samples for three or four days, also increases the total trihalomethane levels produced from a given water (Kissinger and Fritz, 1976). Kissinger and Fritz (1976) postulated that

an exchange reaction could be taking place, whereby organic matter in the water samples reacts with residual chlorine or bromine to produce additional halocarbons. Such halocarbon concentration changes could occur while water is in the distribution system of a city water supply. Bellar et al. (1974) showed that the concentration of trihalomethanes increased as the chlorine contact time was increased up to 15 hours.

METHODS FOR REDUCING TRIHALOMETHANE LEVELS

There are three basic control methods for trihalomethanes in drinking water. These methods are:

1. The use of non-trihalomethane generating disinfectants or alteration of the present method of chlorination so as to reduce the formation of trihalomethanes.

2. Removal or reduction of the precursors prior to chlorination.

3. Removal of the trihalomethanes after formation.

Identification of natural precursors that produce trihalomethanes upon standard chlorination practices will allow improved efforts to remove or reduce these precursors prior to chlorination. Furthermore, once the potential precursors are known, alternative disinfectants or alternative disinfection procedures can be examined. The knowledge of what potential products and amounts of products that can be produced will allow better comparisons between techniques and procedures. It does not make sense to alter an effective established disinfection procedure if the by products produced are equally as hazardous as THM or less hazardous products are formed but effective disinfection suffers.

SECTION 5

MATERIALS AND METHODS

This study investigated algae as potential precursors of trihalomethanes (THM). Algal biomass (cells) and algal extracellular products (ECP) were studied separately for their contribution to THM precursor material. Different phases of algal growth were examined for significant differences in the quantities or types of THM precursors produced. Three species of algae were studied so that significant variations in THM production between algal species could be investigated. Thus, the THM precursor production ability of two basic subdivisions of microorganisms could be compared. Three chlorine doses and two contact times were employed so that the effects of chlorine dose and reaction time might be evaluated, also.

ALGAL SPECIES

The algal species chosen for study were nonaxenic Anabaena cylindrica (UTEX 629), Pediastrum boryanum (UTEX 1372), and Scenedesmus quadricauda (UTEX 614). Anabaena is a member of the Cyanophyta Division, (prokaryotic) while the other two species are Chlorophyta (eukaryotic). The stock cultures were obtained from the Starr Collection at The University of Texas at Austin. These species were selected for study because of their ease of laboratory growth and because of their abundance in South Central Texas lakes as determined from exhaustive literature study.

MEDIA AND CULTURING CONDITIONS

The algae were grown using Bold basal pH 6.6 medium as described by Nichols (1973). The Bold basal medium constituents are given in Table 1. The water used in making the media was obtained from a Millipore Milli-Q Water Purification System which consists of reverse osmosis, two ion exchange cartridges, a super C carbon cartridge and a twin 90 (0.22 μm) filter. This system should remove most of the organics and chlorine demanding substances. The water was boiled with a nitrogen purge for one hour, then boiled after purging for another hour before being used to make the media. After the media preparation the water was autoclaved for 20 minutes at 121°C (15 pounds per square inch) prior to use.

The algae were cultured in Erlemeyer flasks with metal snap-on caps that permitted some air flow to the cultures without allowing contamination. The algae were further aerated by frequent shaking. The glassware used as growth vessels for the algae was cleaned by the following sequential procedure: one wash with Alconox detergent, three tap water rinses, one rinse with H_2SO_4

TABLE 1. Bold Basal pH 6.6 Medium Constituents (Nichols, 1973).

A. Micronutrients - 6 Stock Solutions - use 10 mL each/940 mL water.

Reagent	Concentration (g/400 ml)
$NaNO_3$	10
$CaCl_2.2H_2O$	1
$MgSO_4.7H_2O$	3
K_2HPO_4	3
KH_2PO_4	7
NaCl	1

B. Minor Constituents - 4 Stock Solutions - use 1 mL each/L.

Reagent	Concentration (g/L, unless otherwise noted)
1. EDTA	
EDTA	50
KOH	31
2. Iron	
$FeSO_4.7H_2O$	4.98
H_2SO_4	1.0 mL/L
3. Boron	
H_3BO_3	11.42
4. Micronutrients	
$ZnSO_4.7H_2O$	8.82
$MnCl_2.4H_2O$	1.44
MoO_3	0.71
$CuSO_4.5H_2O$	1.57
$Co(NO_3)_2.6H_2O$	0.49

saturated with $NaNO_3$, four tap rinses, and four organic-free, chlorine demand-free water rinses. The H_2SO_4 saturated with $NaNO_3$ was employed rather than the conventional chromic acid/sulfuric acid cleaning solution because chromic acid leaves a residue that is toxic to some algae. After rinsing, the glassware was dried and sterilized for two hours in a hot air oven at 170°C.

A light source composed of three soft white 20 watt fluorescent bulbs, 24 inches in length was employed. The light emitted was measured at a distance of 14 inches with a General Electric color cosine corrected light meter and the light intensity ranged from 275 to 450 foot-candles from the end of the bulbs to the middle. The algae were grown on a shelf 14 inches from the bulbs and they were subjected to 16 hours of light and eight hours of dark each day on a constant schedule. The temperature for algal growth was 23-25°C.

ALGAL GROWTH MONITORING TECHNIQUES

The techniques used for monitoring the algal growth consisted of dry weight measurements, cell counts, and chlorophyll-a concentration measurements.

Dry weight measurements were made using a Millipore 47 mm diameter filter (9.6 cm^2) vacuum filter apparatus with a 300 mL capacity. Glass fiber filters (5.5 cm diameter) of grade 934AH (Reeve Angel) were used to filter the algae. The filters were first washed with 50 mL of double distilled water to remove any loose filter material, placed in a muffle furnace at 500°C for 10-20 minutes and stored at 100°C until use. Prior to tare weight determination, the filters were placed in a desiccator containing drierite for 30 minutes. The algae were then filtered (10 mL of algal solution) and after filtration, any medium remaining was removed by a wash with 10 mL of distilled water. The filters were dried to constant weight in a 100°C oven and placed in a desiccator for 30 minutes prior to final weighing. Replicate measurements were performed on each organism.

Measurements of chlorophyll-a concentrations were made using a double beam UV-Visible spectrophotometer (Beckman Acta C III) at wavelengths of 750, 663, 645, and 630 nm. Chlorophyll-a was extracted as described in Standard Methods (1975) by first filtering the algae (3-10 mL) through a 0.45 µm membrane filter (Gelman 25 mm) using a small Millipore vacuum filter apparatus. The medium was removed after filtration by a 10 mL distilled water wash. To avoid possible sources of error due to pigment degradation products, 0.2 mL of a magnesium carbonate suspension were added during the final stage of filtering to prevent an increase in acidity and to prevent the stimulation of pheophytin-a (chlorophyll degradation product) in the samples.

The samples were then macerated in a pyrex (15 mL) glass-glass tissue grinder with 5 mL of aqueous acetone solution (90:10 spectrophotometric grade acetone:water). This liquid was poured into a centrifuge tube and steeped overnight in the dark at 4°C. The liquid was then centrifuged for 20 minutes at 2100-2300 rpm (800-1000 x g) (Sorval General Laboratory Centrifuge-2). The solution was protected from light until the absorbance measurements were completed. The extract was transferred to a clean, calibrated, 15 mL screw

cap centrifuge tube and the total extract volume was measured. Control extracts were prepared in exactly the same manner without the algae.

Chlorophyll-a (C_a) was calculated by the following formula (equation 1) where D is the observed optical density at the indicated wavelength:

$$C_a = 11.64D_{663} - 2.16D_{645} + 0.10D_{630} \quad (1)$$

$$C_a \text{ (mg/L)} = \frac{C_a \text{ (volume extract, L)}}{\text{volume sample, L}}$$

An optical density measurement of the extract at 750 nm served as a correction for turbidity. This measurement, when greater than one, was subtracted from each of the pigment optical readings at the other wavelengths before they were used in the calculations. The cuvettes were cleaned after each set of chlorophyll analyses using chromic acid/sulfuric acid cleaning solution and allowing this to sit for 5 to 10 minutes. The cuvettes were rinsed with distilled water and, when feasible, allowed to sit overnight with distilled water. They were then rinsed with spectrophotometric grade acetone and dried with nitrogen gas prior to use. The path length of the cuvettes employed was 1 cm. The spectrophotometer was zeroed on acetone: water (90:10) prior to taking the sample readings.

Spectrophotometric calibration curves for chlorophyll-a were constructed using commericially obtained chlorophyll-a (Sigma Chemical Company) in acetone: water (90:10). Chlorophyll-a dilutions were made and the absorbance for each dilution was measured at 431 and 663 nm (500 dynode volts). A spectral adsorbance curve for chlorophyll-a showed these two wavelengths to be the wavelengths of maximal absorbance. The calibration curves for each wavelength were obtained by plotting the chlorophyll-a concentration versus its absorbance at both wavelengths. Figures 2 and 3 show the spectrophotometric calibration curves and equations 2 and 3 show the regression lines for each wavelength.

$$C_a \text{ (mg/L)} = 11.96D_{663} + 0.0782 \quad (2)$$

$$C_a \text{ (mg/L)} = 10.36D_{431} + 0.0998 \quad (3)$$

The regression lines drawn in both figures had correlation coefficients (R^2) of 0.995. The chlorophyll-a employed was checked for purity by determining the extinction coefficients at the two analytical wavelengths. At 431 nm the determined extinction coefficient was 8.5×10^4 L/mole-cm and at 663 nm it was 7.37×10^4 L/mole-cm. This compared very well with the literature value of 7.50×10^4 L/mole-cm at 663 nm obtained by Meeks (1974) in 80-90% acetone.

Measurements of the chlorophyll-a of the algal samples were also made using a Farrand Optical Company Fluorometer A-4 with a 5 or 60 excitation filter and a 2-28 emission filter. Chlorophyll-a was extracted as described for the spectrophotometric extractions. A calibration curve for the fluorometer (Figure 4) was constructed using commercially obtained chlorophyll-a (Sigma Chemical Company). Dilutions of known chlorophyll-a concentrations were

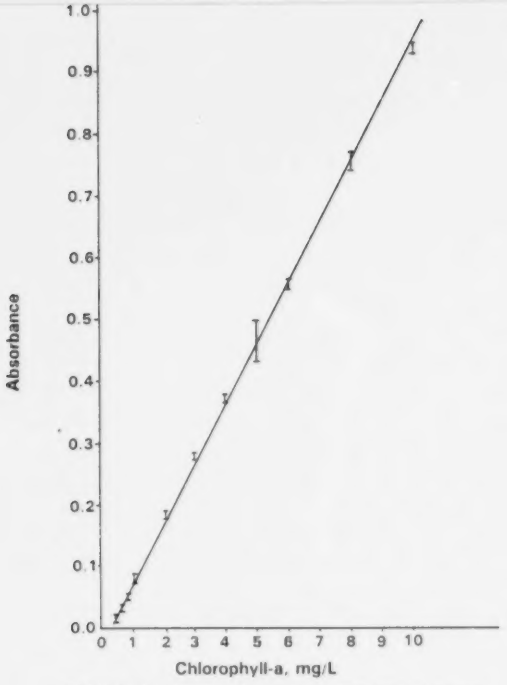


FIGURE 2. Spectrophotometric Calibration Curve for Chlorophyll-a at 431 nm

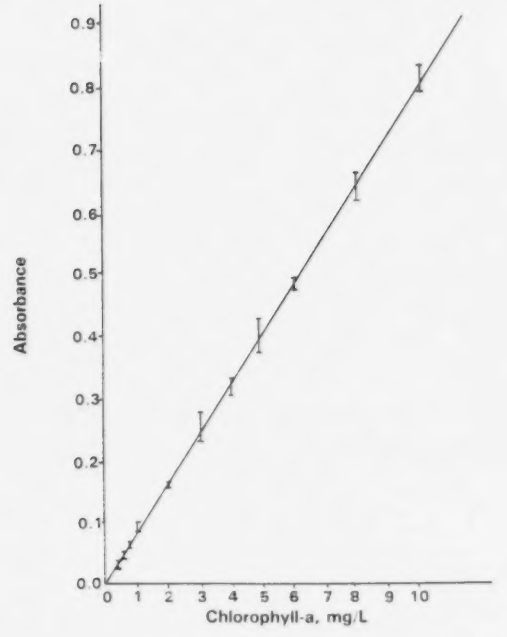


FIGURE 3. Spectrophotometric Calibration Curve for Chlorophyll-a at 663 nm

prepared and the fluorescence of each concentration was plotted against that concentration. The calibration curve is described by equation 4 (R^2 = 0.993):

$$C_a \text{ (mg/L)} = 0.0149 \text{ (reading, range 1)} + 0.0021 \quad (4)$$

The chlorophyll-a concentrations were calculated from algal sample extracts (three-10 mL sample volumes) by measuring their fluorescence and employing the calibration curve to obtain concentration. The fluorometer was initially zeroed on 90:10 acetone:water prior to each day's fluorescence readings. Reference samples were prepared by extraction in the same manner without algae and these readings were subtracted from the fluorescence readings before chlorophyll-a calculations.

Cell count measurements were made along the algal growth curves using a mechanical counter by Coulter Counter (Model ZB1) with an aperture tube of 100 μm diameter. The instrument was calibrated using pollen and glass spheres of known volumes and the optimal sensitivity settings for the measurement of cell counts for each algal species were determined. Figure 5 shows a typical instrument calibration curve for a known particle size (Paper Mulberry pollen of 16 μm size). Sensitivity setting equals the amplification times the aperture current and is related to the volume of the organisms counted. Sensitivity was set so that most of the particles appear 1/2 to 2/3 of the way up the oscilloscope screen. The sensitivity settings employed were: Anabaena, 0.5; Scenedesmus, 8; and Pediastrum, 2. The threshold values used were 5 (lower) and 100 (upper). Commercially obtained electrolyte solution (Coulter Company Isoton® II azide-free) was used for making the cell dilutions (equal volumes of sample and Isoton®) and corresponding control dilutions (medium and Isoton®, equal volumes). The reference reading values made using medium and diluent alone were subtracted prior to cell count calculation. The algae were dispersed by vigorous shaking with glass beads before cell count measurements were performed.

GLASSWARE PREPARATION

All glassware used for reagents, sample preparation, and/or manipulations of any sort that involved THM samples (other than the algal culture vessels themselves) was cleaned by the following sequential procedure: exposure to chromic acid/sulfuric acid cleaning solution for five or more minutes, five or more rinses with organic-free, chlorine demand-free water. The glassware was dried in a 170°C oven prior to use.

CHEMICAL PARAMETER MEASUREMENTS

The chemical parameters measured for the THM samples included: total organic carbon (TOC), total Kjeldahl nitrogen (TKN), ammonia-nitrogen (NH_3-N), pH, temperature, free and total chlorine residuals (one hour and 24 hours chlorine contact time), and THM (measured after one hour and 24 hours chlorine contact time).

The TOC samples were preserved with 0.1 mL of concentrated hydrochloric acid/5 mL of sample and after shaking, were stored at 4°C until time of

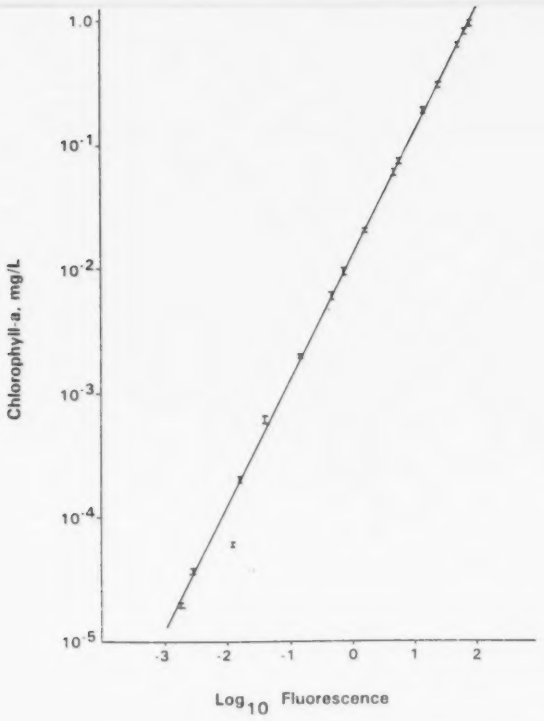


FIGURE 4. Fluorometric Calibration Curve for Chlorophyll-a

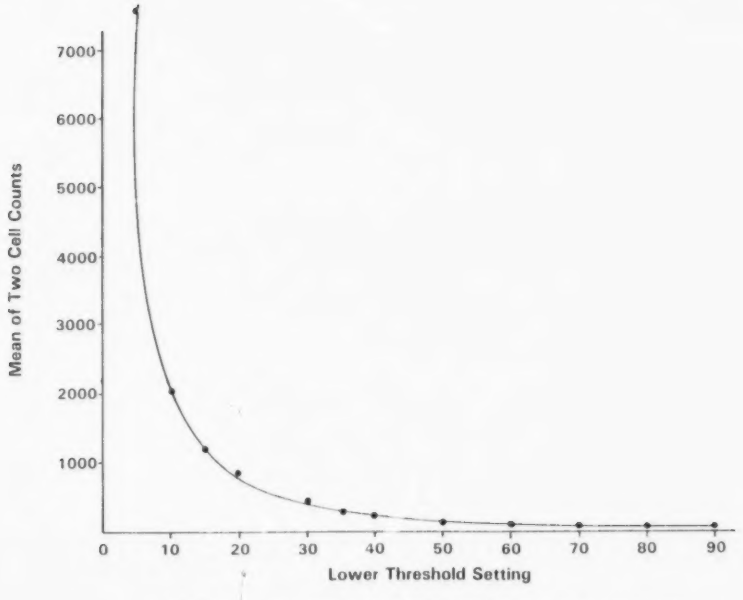


FIGURE 5. Size Distribution of Paper Mulberry Pollen (16 μm) Tube Calibration for 100 μm Aperture Coulter Counter

analysis. After purging the acidified samples with nitrogen gas for approximately three minutes, TOC concentrations were analyzed directly by injection (50 μL) Hamilton SK148-80550-Syringe or an automatic injection syringe) of a 20 μL sample volume into a Beckman Model 915A TOC analyzer. The TKN and NH_3-N samples were preserved with 0.03 mL of a 0.15 molar solution of mercuric chloride per 30 mL of sample and after shaking, were stored at 4°C until analysis. NH_3-N was determined by utilizing the EPA automated alkaline phenolate procedure, modified for low level concentrations, for the Technicon Auto Analyzer® II system. TKN was determined by digestion (modified low level manual EPA and Technicon digestion procedure using a Technicon Block Digestor, DB-20) and dilution to a final constant volume, and analysis on the Technicon Auto Analyzer® II system utilizing a modified alkaline phenolate procedure, adapted from EPA's automated alkaline phenolate procedure for the Technicon continuous digestor.

It was necessary to develop a micro procedure for residual chlorine determinations (free and total) due to the sample size available for analysis. The volume of the cultured algal cells and the volume of the reaction vessels employed were limiting and the use of amperometric titration was not feasible due to the large volumes required for amperometric titrations. Furthermore, the chlorination studies had to be performed in the presence of Bold basal medium (chlorination of the ECP) and a procedure had to be developed that could correct for the influence of metal in the micronutrient solution (e.g., Mn). The leuco crystal violet technique for chlorine residual was modified to use a 5 ml sample (total of 10 ml for both free and total chlorine) in both the presence and absence of the Bold basal medium. The reference technique was amperometric titration (using a Fischer and Porter amperometric titrator Model 17T 1010). The absorbance at 592 nm (500 dynode volts) of the leuco crystal violet color development was monitored on a Beckman UV-Visible Acta C-III spectrophotometer. Calibration curves for free and total available chlorine residual determinations in both chlorine demand-free water and in the Bold basal medium were constructed. Chlorine demand-free water was used in the resuspension of the algal cells for THM precursor studies and the extracellular product THM studies were performed in the Bold basal medium. The free and total chlorine residuals were measured after a one hour chlorine contact time for both the water and the medium calibration curves. The calibration curves obtained for the chlorine demand-free water are shown in equations five and six (R^2 of 0.995 and 0.991, respectively):

$$\text{free chlorine, (mg/L)} = 2.923D_{592} - 0.1223 \quad (5)$$

$$\text{total chlorine, (mg/L)} = 3.765D_{592} - 1.6800 \quad (6)$$

For the Bold basal medium, the calibration curves are shown in equations 7 and 8 (R^2 of 0.941 and 0.985, respectively):

$$\text{free chlorine, (mg/L)} = 4.110D_{592} - 0.6800 \quad (7)$$

$$\text{total chlorine, (mg/L)} = 4.097D_{596} - 1.8920 \quad (8)$$

It was difficult to obtain correlation coefficients closer to one for the Bold basal medium chlorine residual calibration curves because the chlorine residual changes with time. Thus, measurements were made at fixed times and as consistantly as possible. The cuvettes used in making the leuco crystal violet absorbance measurements were cleaned after each series of chlorine tests in the same manner as described for the chlorophyll-a spectrophotometric techniques.

THM were measured using the gas sparging technique developed by Bellar and Lichtenberg (1974). Samples (5 mL) were sparged for 20 minutes at 25°C using 30 mL per minute Grade six helium carrier gas. THM were collected on a Tenax/silica gel trap (30.5 cm long, 0.318 cm OD, 0.293 cm ID, 15.2 cm Tenax, 8.3 cm silica gel) and desorbed at 210°C with a 30 mL per minute helium flow onto a 50°C column (1% SP-1000 on 60/80 mesh Carbopack B, 2.4 m long, 0.318 cm OD, 0.216 cm ID). The column was held isothermally at 50°C for six minutes and temperature programmed to 200°C at a rate of 10°C per minute. All analyses were performed on a Varian 3700 gas chromatograph with a flame ionization detector system and a CDS 111C reporting integrator.

SECTION 6

EXPERIMENTAL PROCEDURE

Algae from a stock culture with a known cell/mL concentration were inoculated into two liters of freshly prepared medium to make a final dilution of approximately 10^3 cells/mL. The algae were then cultured until the growth phase desired for THM analysis was reached. The growth phases desired for THM precursor studies were determined by cell count techniques and chlorophyll-a measurements. These growth monitoring techniques allowed the algal growth curves to be generated. From these curves the times chosen for THM precursor examination were selected with the intention of encompassing the five basic growth phases of each species employed. These basic growth phases are: lag, early exponential, late exponential, early stationary, and late stationary. The death phase was not examined. Spectrophotometric measurements of the chlorophyll-a and the plotting of this data allowed the extraction techniques to be perfected and provided preliminary growth curves (higher initial cell/mL concentrations than 10^3 cell/mL) for each species. Cell counts and fluorometric assay of each species' chlorophyll-a were the final methods chosen for the production of the growth curves that allowed the selection of the THM experimentation times. The fluorometric chlorophyll-a technique showed less data scatter and was more sensitive to low levels (i.e. μ g/L) of chlorophyll-a than the spectrophotometric chlorophyll-a assay. The two preliminary chlorophyll-a growth curves produced by spectrophotometric assay are shown in Figures 6 and 7. The growth curves generated by fluorometric assay of chlorophyll-a for each algal species are illustrated in Figures 8 to 10. The cell count growth curves employed for deriving the THM experimentation times for each species are shown in Figures 11 to 13. The times chosen for experimentation along the growth curve of Anabaena were days 0, 2, 4, 7, 10, 14, and 21. Day 0 encompassed the brief, if any, lag phase; days 2, 4, and 7 were during the exponential growth phase, and day 10 was transitional between the late exponential and early stationary phases. Days 14 and 21 were during the stationary phase. Similarly, for Pediastrum, days 0, 2, 7, 10, 15, 21, and 28 were chosen for study and Scenedesmus experiments were conducted on days 0, 2, 4, 8, 11, 15, and 21 of the growth cycle.

At the chosen time, an aliquot of 270 mL was removed from the two liter initial culture. From this aliquot, 20 mL were used for growth monitoring measurements done by cell enumeration and fluorometric assay of the chlorophyll-a. Fluorometric chlorophyll-a measurements and cell counts were made on the algae each time that THM experiments were performed. The cell count technique was performed using a 5 mL volume of the sample diluted with 5 mL of Isoton®. This blank reading of the Coulter Counter was then subtracted from the algal cell counts before cell count calculations. The algal cell

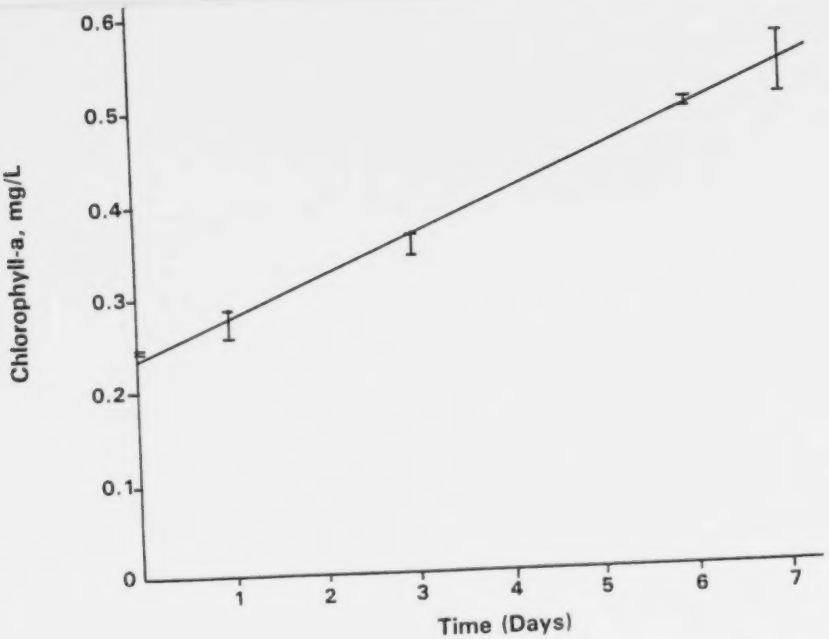


FIGURE 6. Anabaena Growth Curve - Chlorophyll-a Spectrophotometric Determination

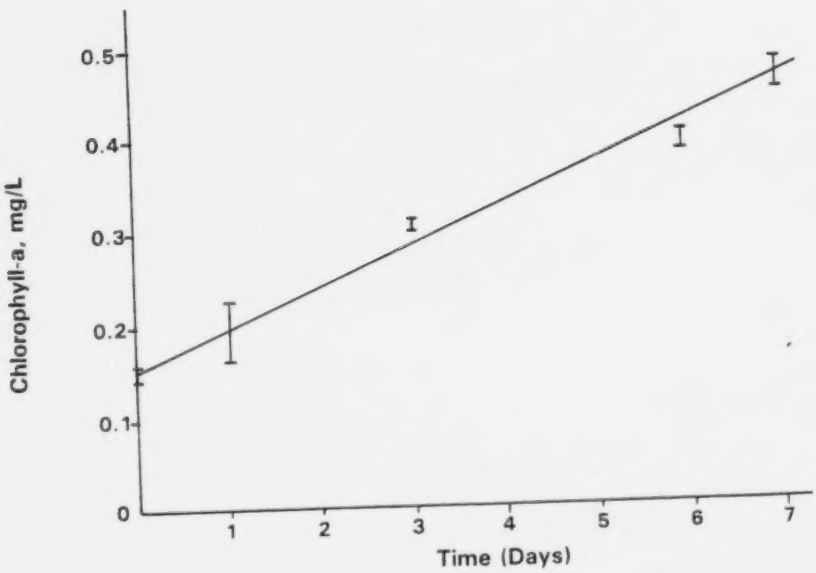


FIGURE 7. Scenedesmus Growth Curve - Chlorophyll-a Spectrophotometric Determination

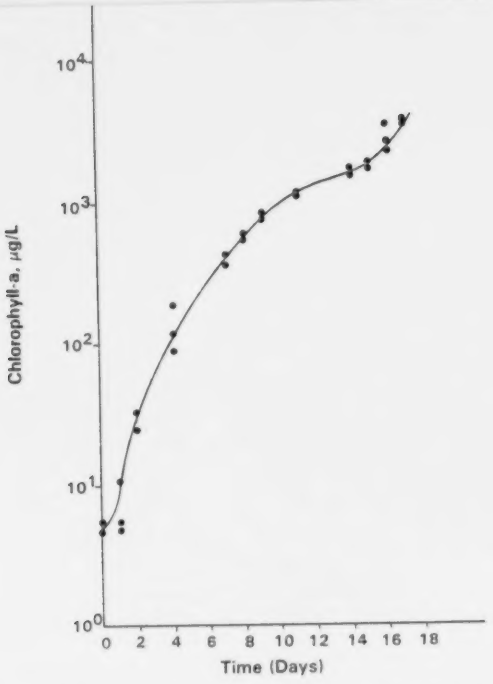


FIGURE 8. Anabaena Growth Curve - Chlorophyll-a

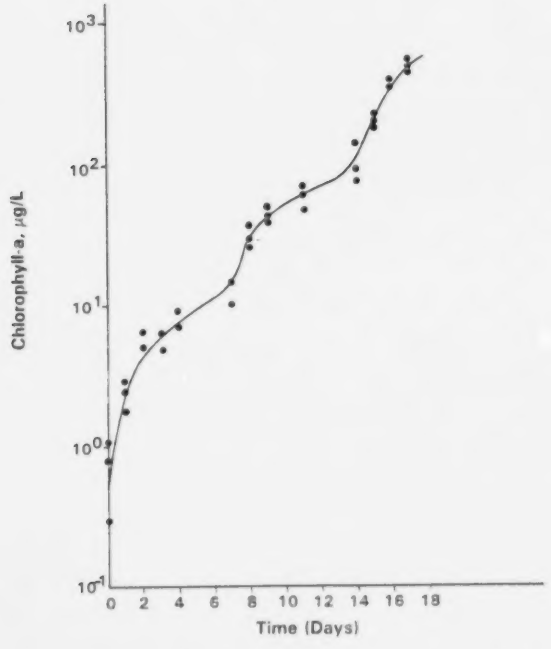


FIGURE 9. Pediastrum Growth Curve - Chlorophyll-a

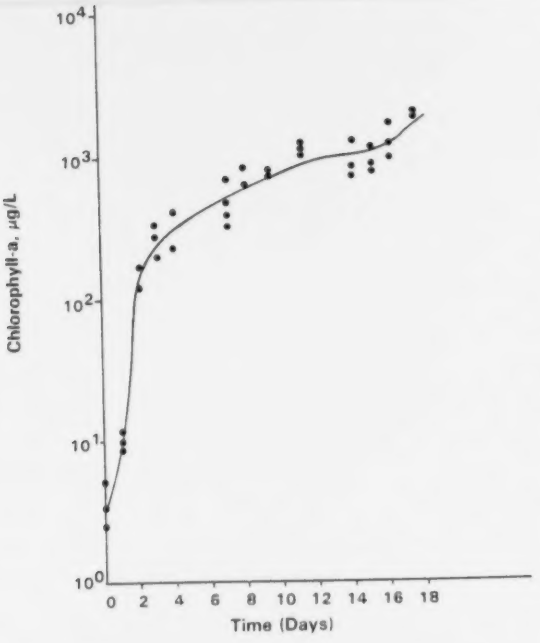


FIGURE 10. Scenedesmus Growth Curve - Chlorophyll-a

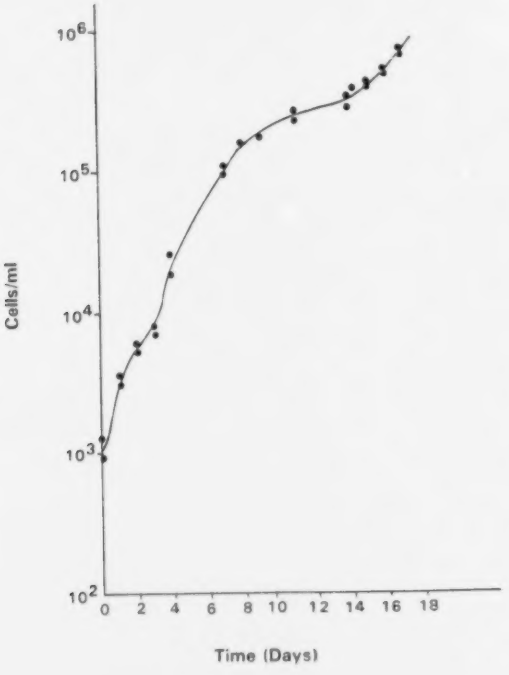


FIGURE 11. Anabaena Growth Curve - Cell Count

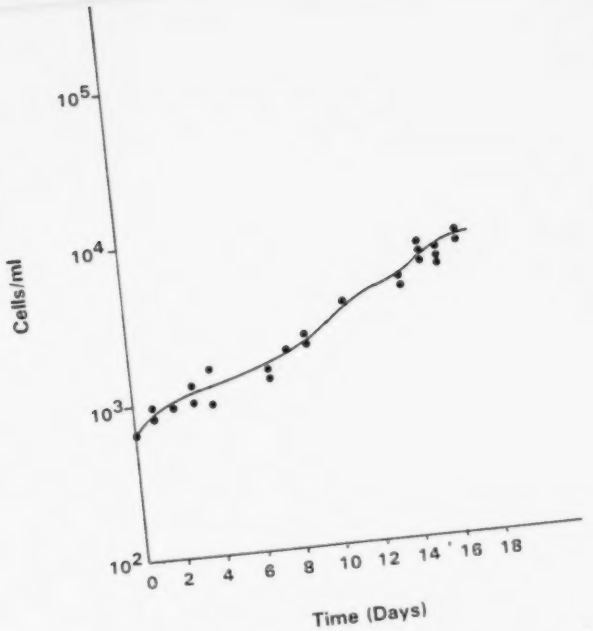


FIGURE 12. Pediastrum Growth Curve - Cell Count

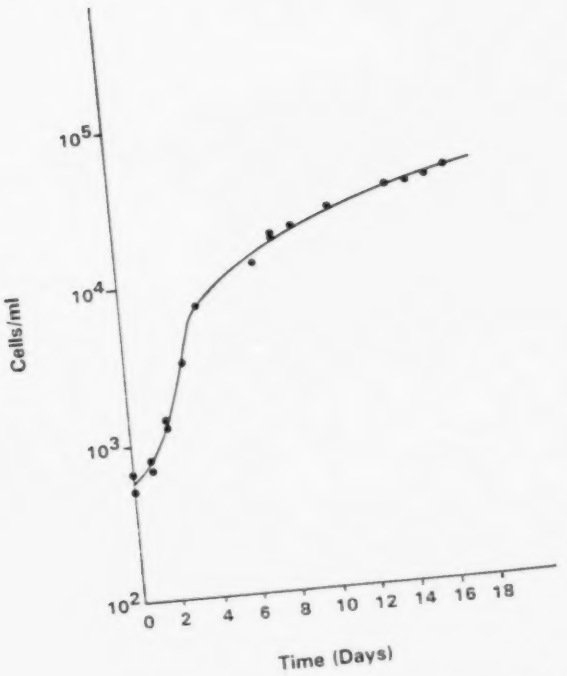


FIGURE 13. Scenedesmus Growth Curve - Cell Count

count samples were done in duplicate. The chlorophyll-a extractions for the fluorometric assay were also done in duplicate employing a 5 mL sample volume for each. Fluorometric reference samples were extracted in the same manner except that 5 mL of distilled water were used rather than the algal sample.

Dry weight measurements of the algae were not employed in the growth monitoring procedures finally adopted. These measurements were abandoned because there was troublesome data scatter of the results due to the low amounts of material weighed. The filters employed were modified from the use of 5.5 cm glass fiber filters (as previously described) to the use of 27 mm diameter filters (Reeve Angel) with only slightly improved results. Dry weight curves for Scenedesmus using both the 5.5 cm and the 27 mm filters are given in Figures 14 and 15. The standard deviation for a single 20 mL sample of Scenedesmus (fifteen replicates) was observed to be ± 10 mg/L even when a relatively high initial volume was employed. A much larger algal sample would improve the results; however, there were severe volume limitations imposed by the chlorination experiments and THM analyses. In order to perform all the tests for growth monitoring and for measurement of all the chemical parameters, the required initial volume of the algal sample was already quite large. Increasing this initial volume to the point where accurate dry weight measurements could be performed would require an impractical volume in regard to the growth space available and in regard to ease of handling. The use of chlorophyll-a measurements and cell counts by Coulter Counter techniques rectified the imprecision of the dry weights and allowed comparison between the techniques.

After the growth monitoring measurements were made, 250 mL of the 270 mL algal aliquots remained. This volume was used for the THM precursor experiments and was separated into the cells and their corresponding extracellular products by centrifugation (Sorval) at 2400-2600 rpm (1050-1250 x g) for 20 minutes. The ECP and medium (supernatant) were then poured off and vacuum filtered using a 0.45 μm membrane filter (47 mm). The filtered ECP were transferred to a clean flask and refrigerated (4°C) in the dark overnight or until chlorine addition (usually the same or next day). Prior to chlorination, the ECP were warmed to room temperature. The pellet formed after centrifugation (algal biomass) was washed twice with 0.9 percent sterile sodium chloride (Travinol®), resuspended, recentrifuged, and the supernatant discarded. An isotonic salt wash was employed rather than a water wash to prevent lysing of the cells before chlorine addition. The cells, after removal of the Travinol®, were resuspended in 250 mL of organic-free, chlorine demand-free water (made in the same manner as the water employed in making media). Approximately 10^{-4} moles of phosphate buffer (filtered through a 0.45 m membrane filter) were added to the resuspended cells for pH control. The resuspended cells or ECP aliquots were then divided into three 80 mL samples, the pH and temperature measured, and immediately each sample was dosed with the desired amount of chlorine. The chlorine doses employed are shown in Table 2. After one hour of chlorine contact time, the pH, temperature and chlorine residuals (free and total) were again measured in duplicate. Samples were obtained for TOC, TKN, and NH_3-N determinations. A THM sample (one hour contact time) was collected in a

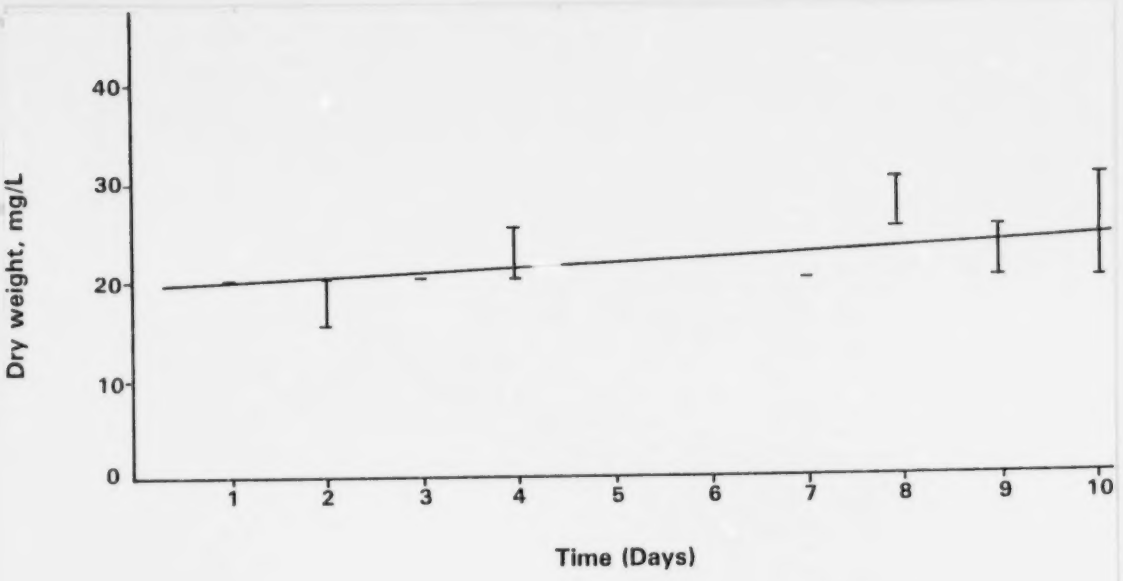


FIGURE 14. Scenedesmus Growth Curve - Dry Weight
5.5 cm filters

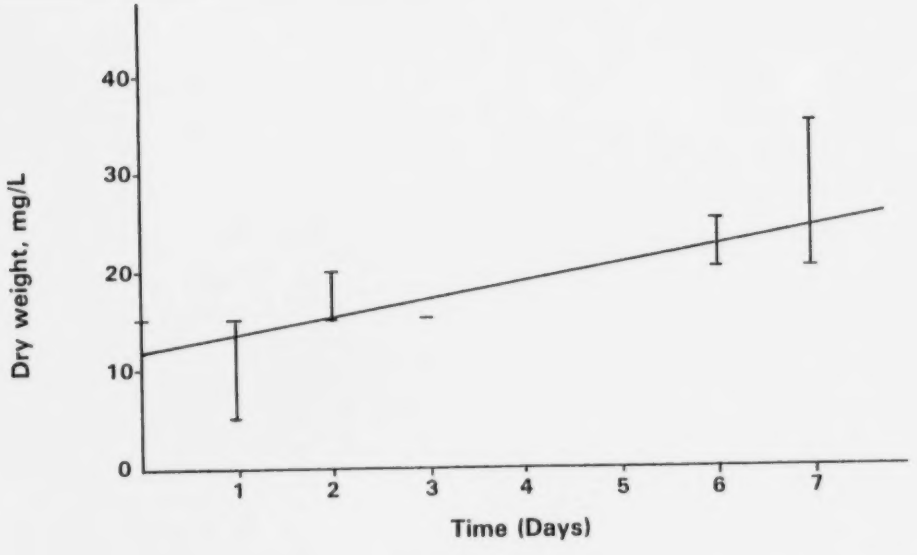


FIGURE 15. Scenedesmus Growth Curve - Dry Weight
27 mm filters

TABLE 2. Chlorine Doses Employed for Extracellular Products (ECP) and Resuspended Cells of *Anabaena*, *Scenedesmus*, and *Pediastrum*

Sample	Chlorine Dose mg/L
Anabaena Cells	1.5
	5.0
	9.0
Anabaena ECP	6.8
	23.6
	32.5
Scenedesmus Cells	1.5
	5.0
	9.9
Scenedesmus ECP	6.8
	23.7
	32.6
Pediastrum Cells	1.5
	5.0
	9.9
Pediastrum ECP	6.8
	23.7
	32.6

vial (10 mL) containing 0.3 to 1.0 mL of 0.01 N sodium thiosulfate (according to chlorine dose) to quench the reaction (instantaneous THM) and sealed with a teflon-faced septum that allowed no headspace. A second THM sample was stored at room temperature in a teflon sealed vial with no headspace for 24 hours and then quenched with sodium thiosulfate (terminal THM). After sodium thiosulfate addition the THM samples were all stored at 4°C until analysis. All vials were warmed to room temperature prior to THM analysis.

Negative controls were prepared in the same manner as the samples. The cell controls were prepared using a 250 mL aliquot of the same batch of organic-free, chlorine demand-free water as that used to resuspend the cells. The same amount of phosphate buffer (10^{-4} moles) was added and the aliquot was divided into three 80 mL samples for chlorine dosing with the same chlorine levels as employed for the cells. The ECP controls were prepared from a 250 mL aliquot of the same batch of Bold basal medium as that which was used for culturing of the algae. The medium was filtered (as the ECP were filtered) and the filtrate was divided into three 80 mL samples. These three samples were then chlorine dosed with the same doses as those employed for the ECP. The pH, temperature, chlorine residuals (free and total), TOC, TKN, NH_3-N, and THM (both one hour and 24 hour) of the reference samples (both medium and water) were determined in the same manner as for the cells and ECP. The medium and water reference sample sets were prepared twice during the growth cycle of each organism studied. One set of controls (both medium and water) was prepared shortly after the initiation of each species' growth curve (inoculation of algae). The second set of controls (both medium and water) was completed after the growth cycle experiments were terminated. These references were checks on any THM precursor materials that might be absorbed by the water or medium from the atmosphere during the three weeks, or so, that the algal growth curve experiments were in progress and served as correction blanks for the chemical parameters measured.

A flow chart illustrating the experimental procedure for Anabaena is given in Figure 16. Scenedesmus and Pediastrum were handled in precisely the same manner for all the steps in the flow chart except for the experimental times chosen from their growth curves.

Additional references (both water series and medium series) were prepared after all the algal experiments were completed. These references were used to determine if any of the reagents or solutions (i.e., sodium thiosulfate, etc.) were contaminated with THM precursor materials. THM analyses were performed on these samples within two weeks.

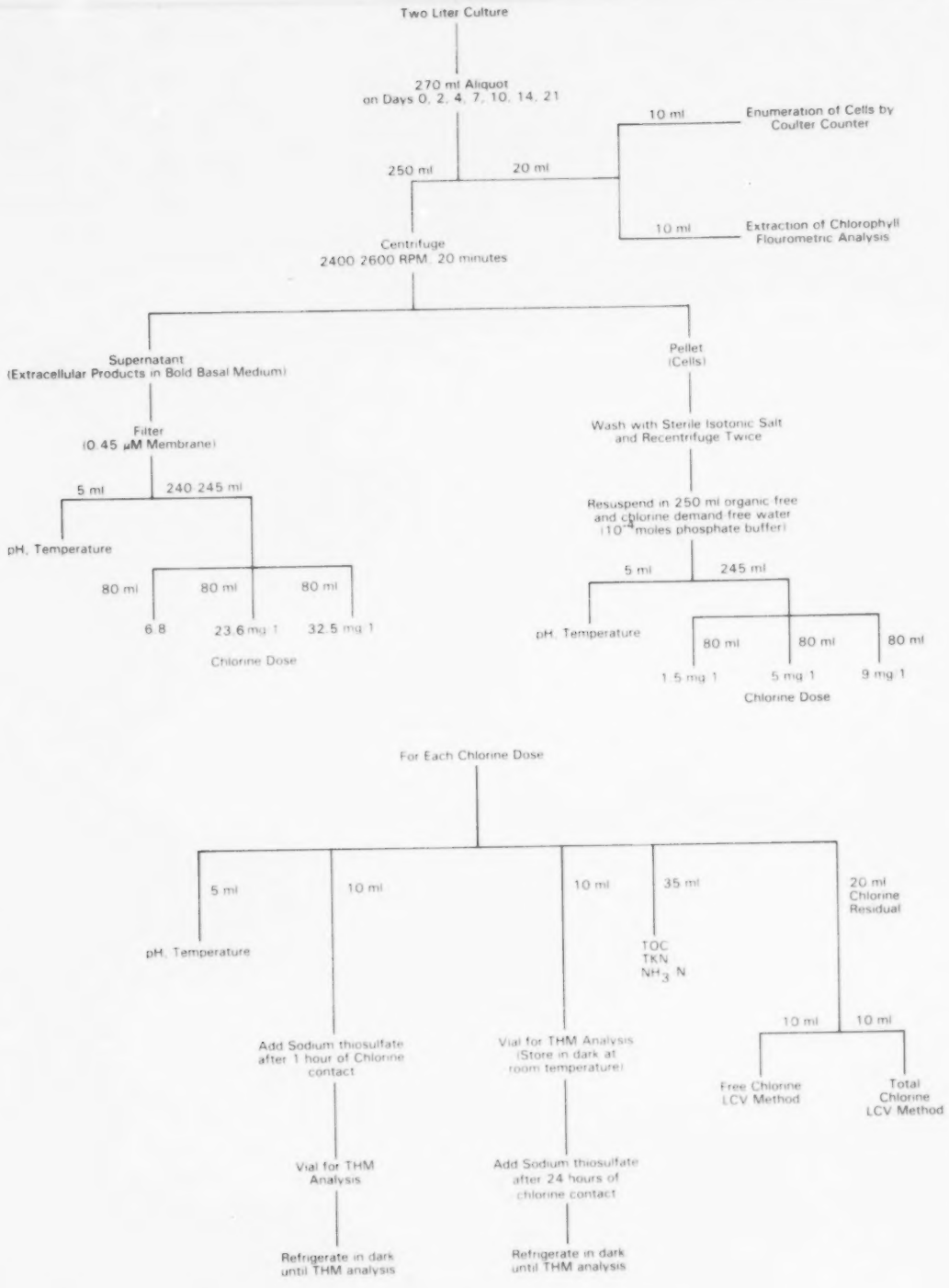


FIGURE 16. Procedural Flow Chart for Algae Studies with Anabaena

SECTION 7

RESULTS

The relationship between chlorophyll-a as an indicator of algal biomass and cell count determined by mechanical counting for the growth cycles of the three algal species examined in this study is illustrated by Figures 17 to 19. Increasing cell count was directly related to the amount of chlorophyll-a produced by the cells. Linear regression analyses were done for each algal species by averaging each day's chlorophyll-a replicate measurements and comparing these with the average of that day's cell count replicate measurements. Linear regression analyses yielded linear correlation coefficients (R^2) for chlorophyll-a to cell count of each species. The R^2 obtained for Anabaena was 0.952; Scenedesmus was 0.955; and Pediastrum was 0.989. For a given species, growth curves generated from both techniques of growth measurement gave the same shape curves. The three species exhibited different growth curve shapes and growth rates. The growth curves generated for all three species using both monitoring techniques are given in Figures 20 to 25 for the experiments in which THM levels were measured.

The chemical parameters TOC, TKN, and NH_3-N for the cells of all three species increased in a manner similar to that of the cell count and chlorophyll-a as the organisms aged (Figures 26 to 31). (In all the figures, arrows pointing downward from a data point indicate that the level of the measured parameter was less than the detection limit of the technique employed to measure that paramater). However there was little change in TOC, TKN, and NH_3-N from the isolated Anabaena and Pediastrum cells until the tenth day of growth. The Scenedesmus isolated cells began to show increases in TOC, TKN, and NH_3-N as early as the fourth day of growth. On the other hand, TOC and NH_3-N exhibited a constant level or drop in value for the ECP isolated from Anabaena with age of culture. TKN for Anabaena isolated ECP dropped initially and then began a slight increase around day 10 of the growth curve. However, later in the growth curve, the levels seemed to decline again. The TKN and NH_3-N levels from the Pediastrum ECP climbed with culture age until day 21 of growth at which time the TKN and NH_3-N levels declined. The TOC levels for the Pediastrum ECP were variable. Initially the levels were relatively high but then exhibited a dip during the middle portion of the growth curve. The TOC levels began to climb toward the later phases of growth. For the ECP isolated from Scenedesmus the NH_3-N levels remained constant or declined with age of culture. TOC and TKN levels from the Scenedesmus ECP initially increased with culture age until day four or five of growth at which point the TKN levels began to decline slightly with culture age while the TOC levels began to increase toward the end of the growth curve. The TOC, TKN, and NH_3-N

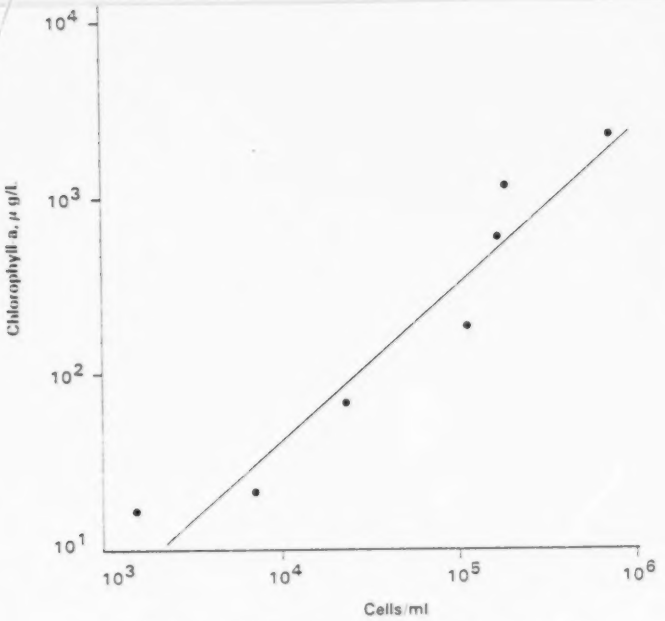


FIGURE 17. Anabaena Cell Count Versus Chlorophyll-a for Days 0 through 21 of Growth

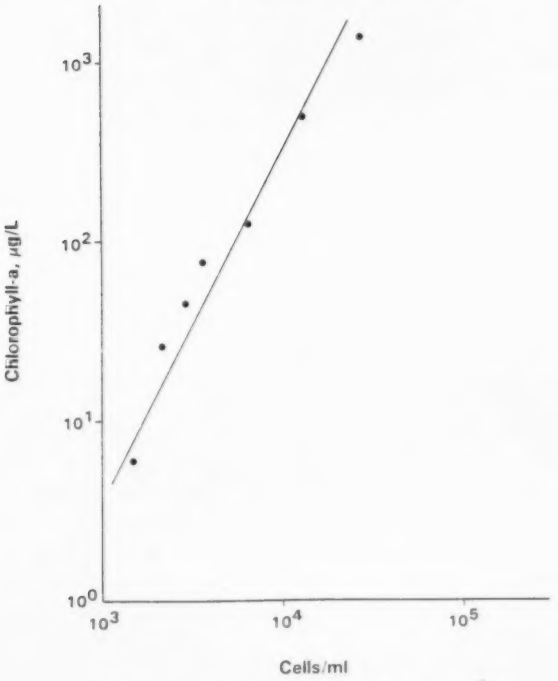


FIGURE 18. Pediastrum Cell Count Versus Chlorophyll-a for Days 0 through 28 of Growth

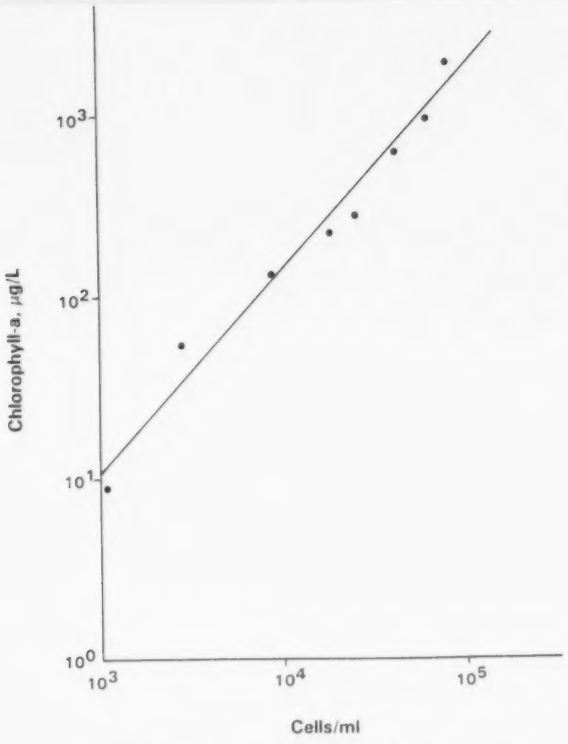


FIGURE 19. Scenedesmus Cell Count Versus Chlorophyll-a for Days 0 through 21 of Growth

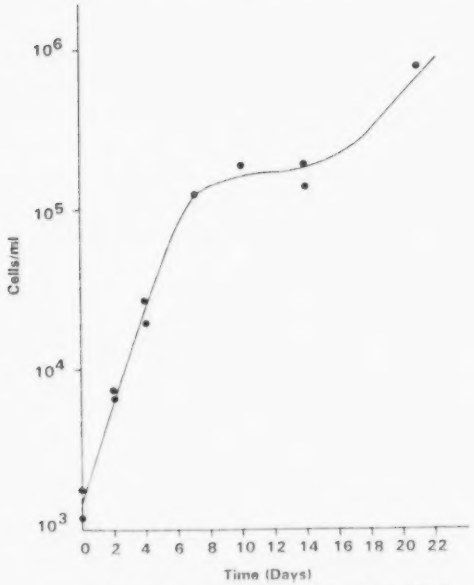


FIGURE 20. Anabaena Growth Curve Cell Count

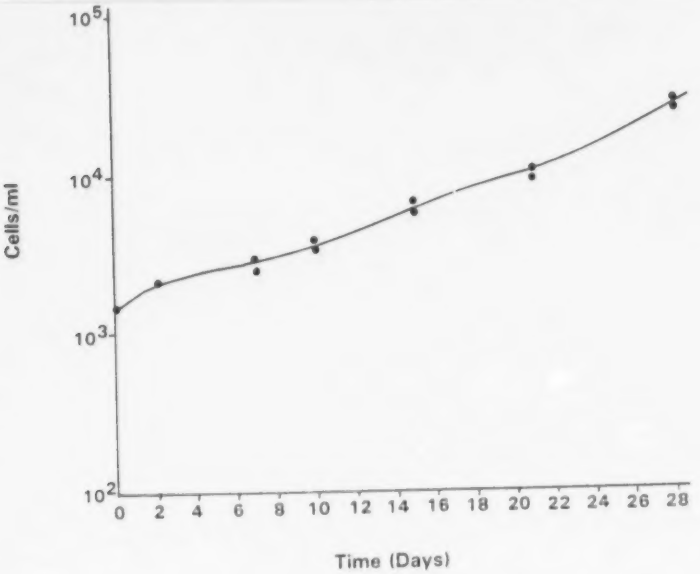


FIGURE 21. Pediastrum Growth Curve - Cell Count

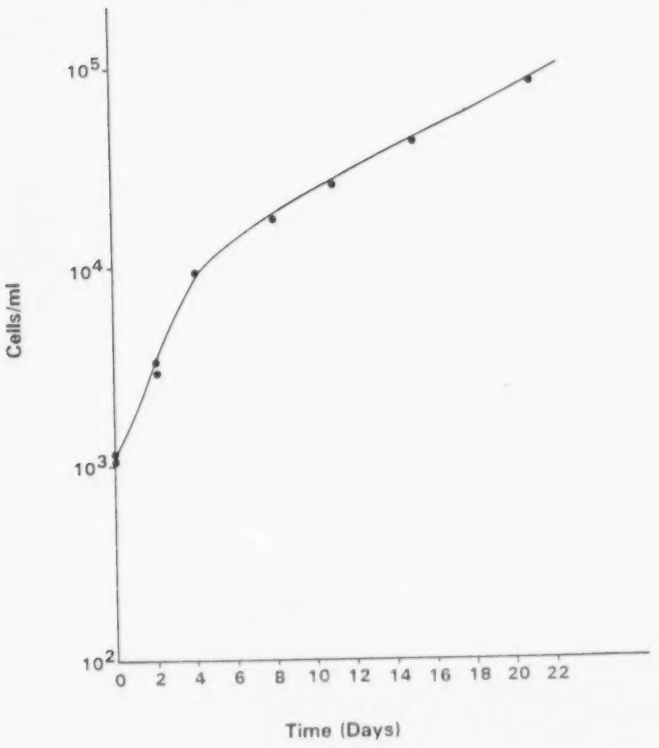


FIGURE 22. Scenedesmus Growth Curve - Cell Count

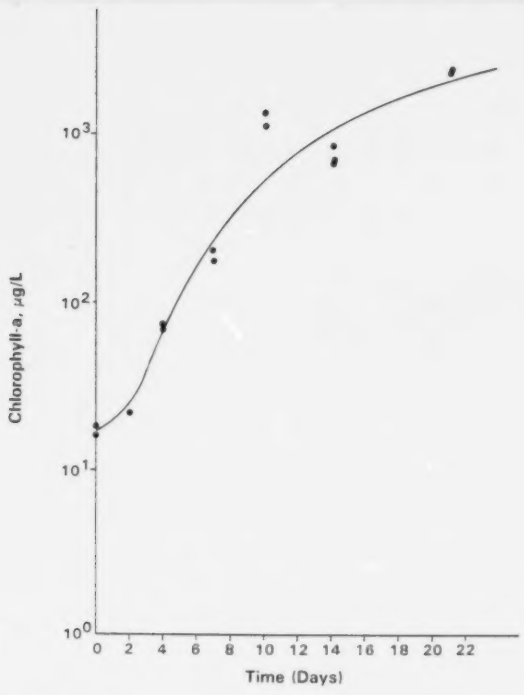


FIGURE 23. Anabaena Growth Curve - Chlorophyll-a

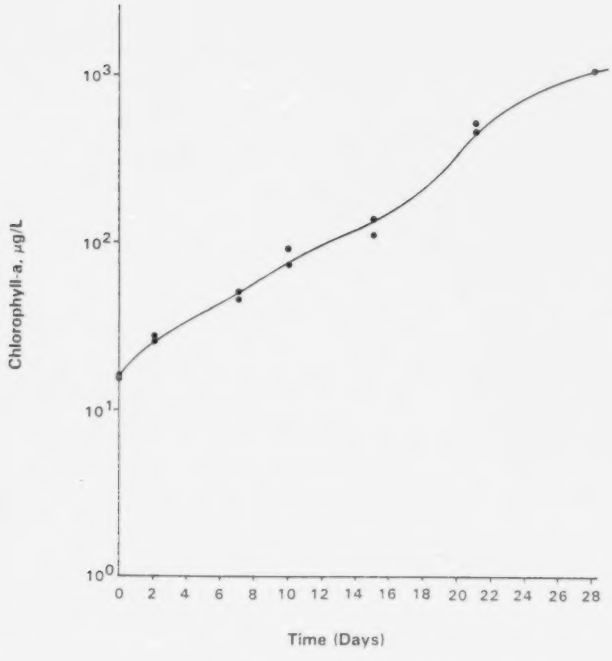


FIGURE 24. Pediastrum Growth Curve - Chlorophyll-a

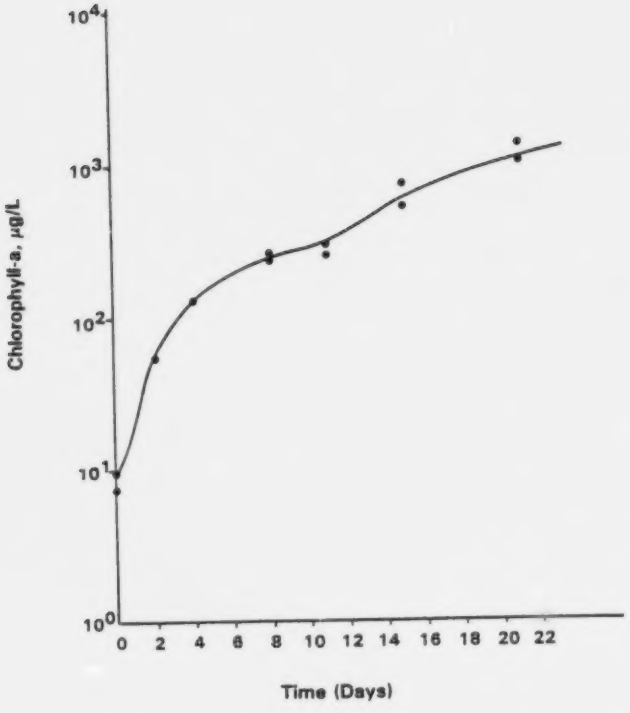


FIGURE 25. Scenedesmus Growth Curve - Chlorophyll-a

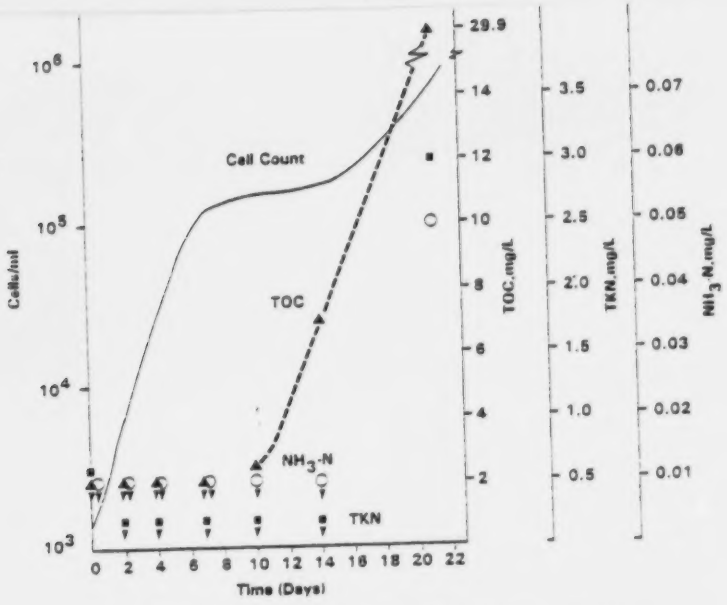


FIGURE 26. Variations in TOC, TKN, NH_3-N and Cell Count for Anabaena Cells 9.0 mg/L Chlorine Dose Versus Age of Culture

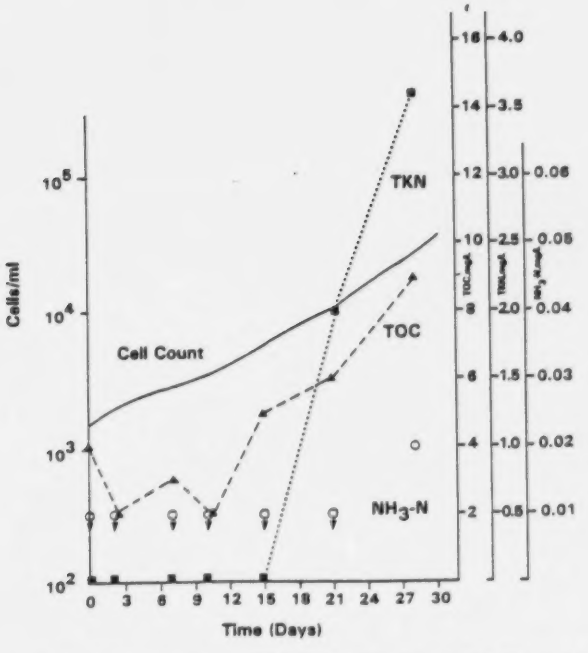


FIGURE 27. Variations in TOC, TKN, NH_3-N and Cell Count for Pediastrum Cells 9.9 mg/L Chlorine Dose Versus Age of Culture

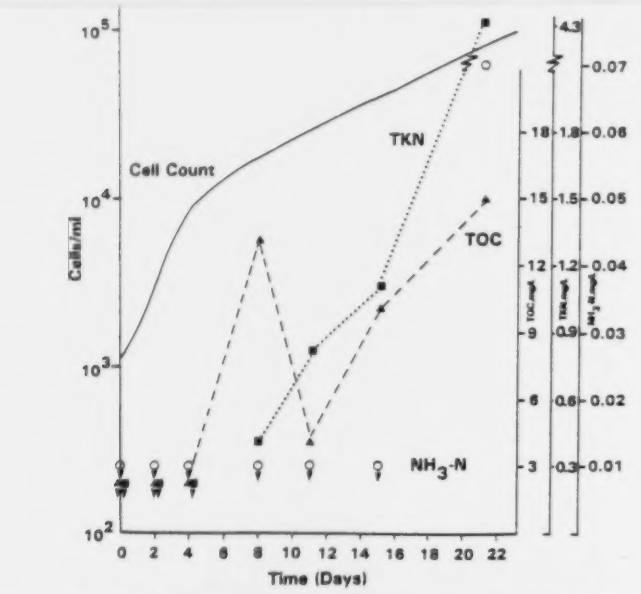


FIGURE 28. Variations in TOC, TKN, NH_3-N and Cell Count for Scenedesmus Cells 9.9 mg/L Chlorine Dose Versus Age of Culture

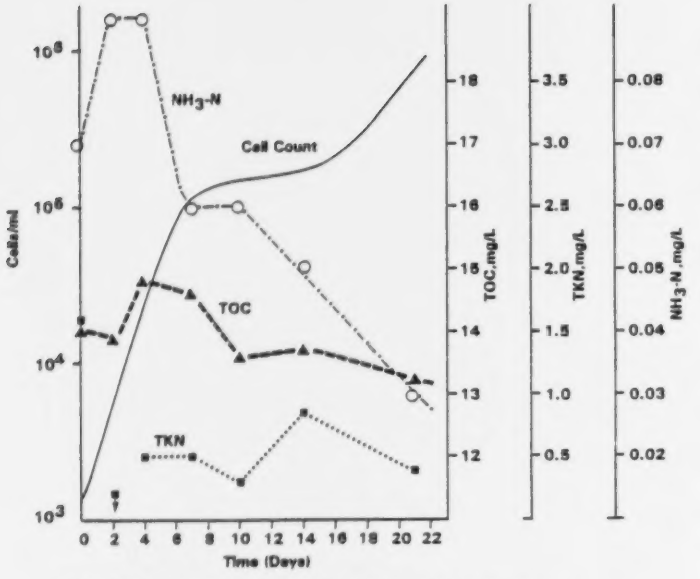


FIGURE 29. Variations in TOC, TKN, NH_3-N and Cell Count for Anabaena ECP after 23.6mg/L Chlorine Dose Versus Age of Culture

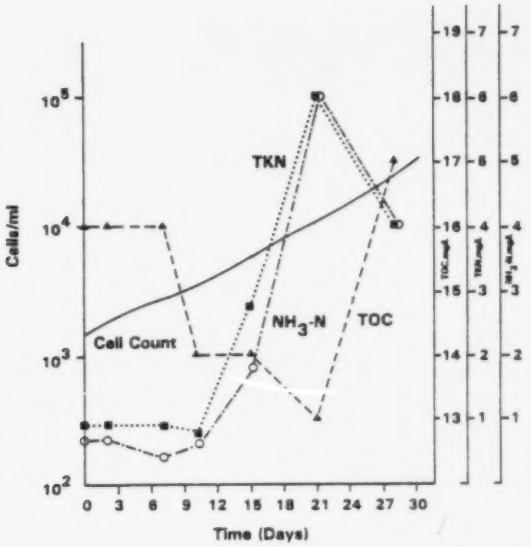


FIGURE 30. Variations in TOC, TKN, NH_3-N and Cell Count for Pediastrum ECP 23.7 mg/L Chlorine Dose Versus Age of Culture

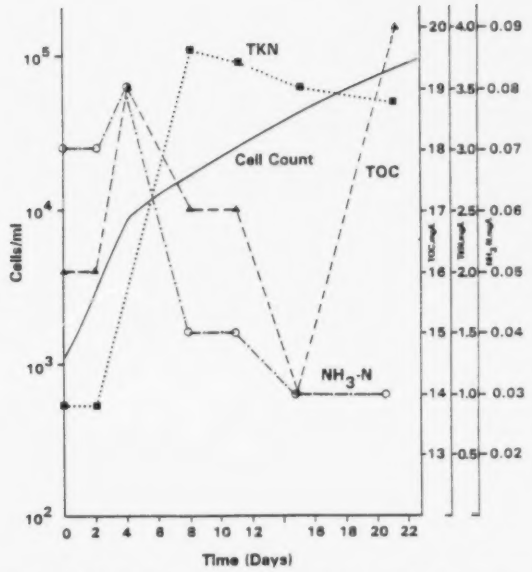


FIGURE 31. Variations in TOC, TKN, NH_3-N and Cell Count for Scenedesmus ECP 23.7 mg /L Chlorine Dose Versus Age of Culture

values for chlorine doses not shown exhibit the same general trends observed in Figures 26-31 for the various algae species. Separate control experiments established that there was no effect, within experimental error, of chlorination on these parameters.

After adding chlorine to the cell and ECP test samples of Anabaena THM production was monitored at contact times of one and 24 hours. The pH for all measured data points ranged between 6.5 and 7.4 for the ECP and 6.4 and 7.6 for the cells. The temperature ranged between 21 and 24°C for all Anabaena measurements. Total THM (TTHM) production from Anabaena ECP at various times in the growth curve is demonstrated in Figures 32 to 34. For a one hour contact time and a chlorine dose of 23.6 mg/L (Figure 32), a typical effect was observed. Total THM production increased slightly and then falls off as the stationary phase of growth was reached. The free chlorine residual was essentially zero for the low doses during the early portions of the growth curve indicating a high chlorine demand. After six days, however, a free residual persisted indicating a change in the type and/or degree of ECP produced. The 32.5 mg/L dose (Figure 33) showed the same free chlorine residual trend as that shown for the 23.6 mg/L dose (Figure 32) except with a slightly higher residual. The total THM production from a 24 hour contact time for the 23.6 mg/L chlorine dose on the ECP of Anabaena is not shown because a free chlorine residual was not apparent. However, THM production increased with an ill-defined maximum near the late exponential growth phase followed by a slight decrease in amount of THM. This type pattern was observed for the other chlorine doses. The drop off in THM level was not due to chlorine limitations alone since a free residual (0.4 mg/L) persisted for the 32.5 mg/L dose and a similar decrease was noted (Figure 34).

Results of the chlorination of the isolated Anabaena cells paralleled the results observed for the ECP. A free chlorine residual was maintained throughout the growth cycle except in the case of the lowest dose (at days in the growth cycle greater than 10). Maximal levels of THM produced by the Anabaena cells compared to ECP on selected days of the growth cycle are given in Table 3.

The Scenedesmus cells exhibited a pH range of 6.2 to 7.4 while the pH of the ECP ranged from 6.4 to 7.0. The temperature for all Scenedesmus measurements ranged from 24 to 25°C. The chloroform production from the Scenedesmus cells and ECP (selected doses and contact times) is shown in Figures 35 to 37. The Scenedesmus cells, both after one and 24 hours of chlorine contact time showed high chloroform production immediately after initiation of the experiment. After the initially high levels, the chloroform levels dropped until approximately day 4 of growth at which time a peak in chloroform production was observed (days 4-10). After this peak in chloroform production the chloroform levels dropped to less than the detection limits towards the last day examined along the growth curve (day 21). The Scenedesmus ECP, one hour contact time, produced chloroform levels in generally the same pattern as that described for the cells. For the Scenedesmus ECP after 24 hours of chlorine contact time, maximal chloroform production occurred somewhat later in the growth curve (approximately days 11-21) than for the cells of the one hour contact time ECP.

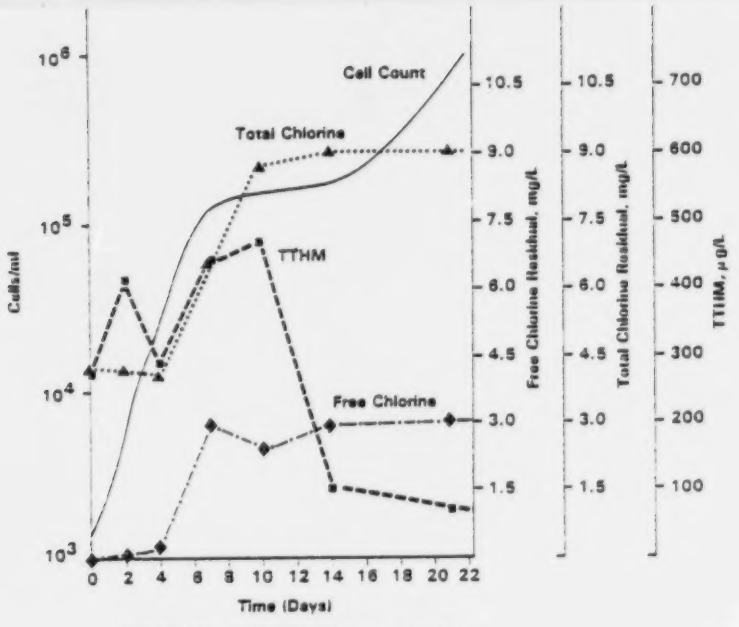


FIGURE 32. THM Production from Anabaena
ECP, 23.6 mg/L Chlorine
Dose, 1 Hour Contact Time

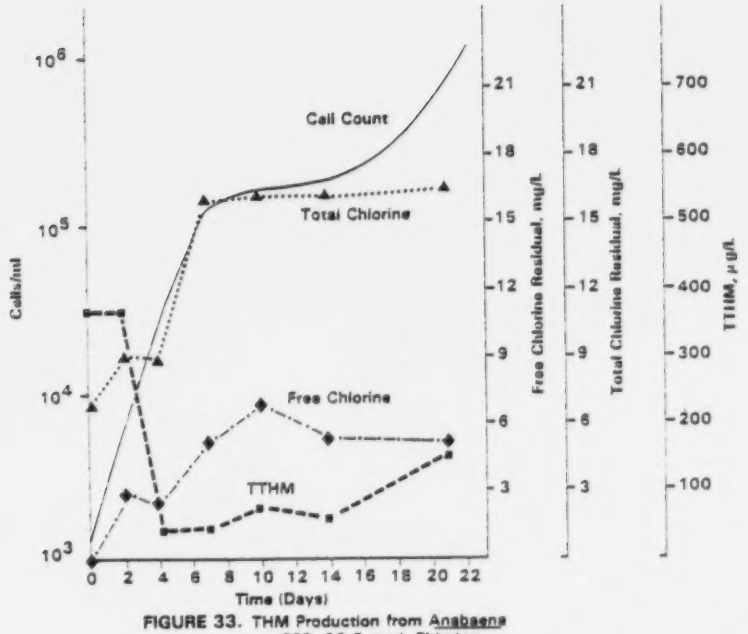


FIGURE 33. THM Production from Anabaena
ECP, 32.5 mg/L Chlorine
Dose, 1 Hour Contact Time

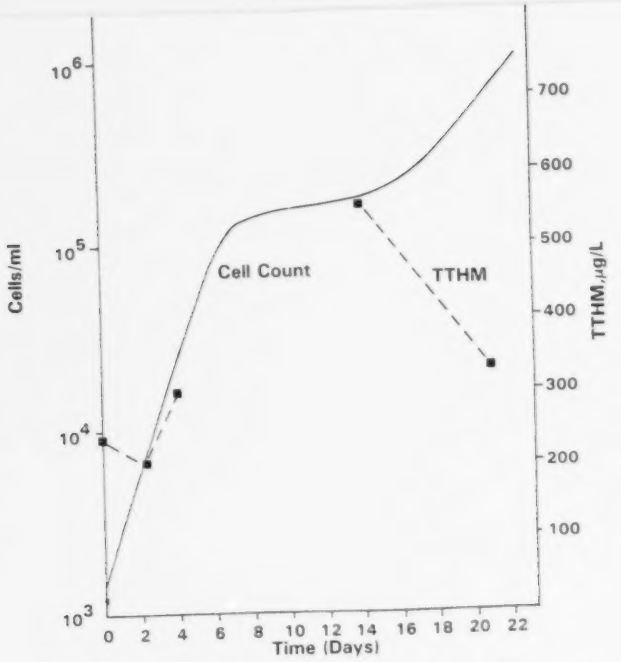


FIGURE 34. THM Production from Anabaena ECP, 32.5 mg/L Chlorine Dose, 24 Hours Contact Time

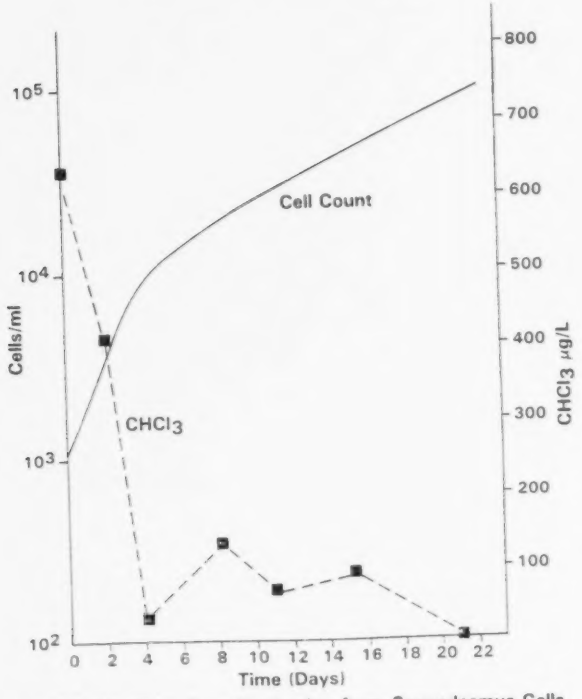


FIGURE 35. Chloroform Production from Scenedesmus Cells, 5.0 mg/L Chlorine Dose, 1 Hour Contact Time

TABLE 3. Comparison of Trihalomethane Production from Anabaena Cells and Extracellular Products at Selected Times in the Growth Cycle

Day in Growth Cycle	Contact Time (Hrs)	Cells* Chlorine Dose (mg/L)	Cells* Free Chlorine Residual (mg/L)	Cells* TTHM (µg/L)	ECP Chlorine Dose (mg/L)	ECP Free Chlorine Residual (mg/L)	ECP TTHM (µg/L)
7	1	1.5	0.6	315.6			
	1	9.0	6.1	392.8	32.6	4.1	45.4
21	1	4.9	0.1	138.0	23.6	<0.1	73.1
	1	8.7	4.6	392.8	32.5	<0.1	149.5
	24	-	-	-	32.5	0.4	332.0

*Cells collected by centrifugation from the initial aliquot were resuspended in a volume of water equal to the volume of ECP collected.

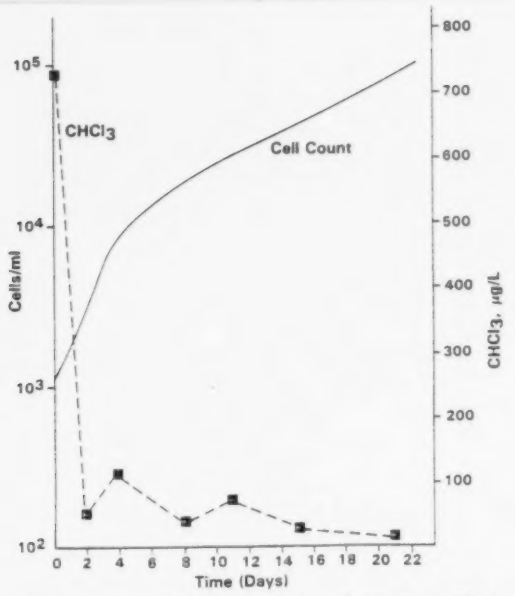


FIGURE 36. Chloroform Production from Scenedesmus ECP, 32.6 mg/L Chlorine Dose, 1 Hour Contact Time

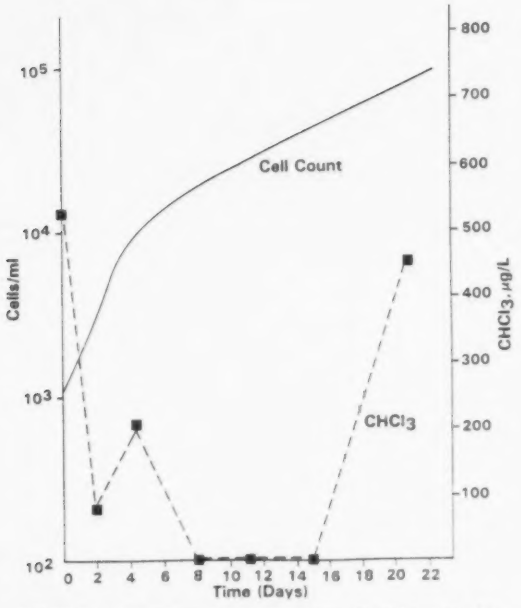


FIGURE 37. Chloroform Production from Scenedesmus ECP, 32.6 mg/L Chlorine Dose, 24 Hours Contact Time

The Pediastrum cells had a pH range of 6.5 to 7.4 while the pH of the ECP ranged from 6.5 to 7.9. For the Pediastrum cells and ECP the temperature ranged between 23 to 25°C. Chloroform production from the Pediastrum cells and ECP (selected doses and contact times) is shown in Figures 38 to 40. For the Pediastrum cells, after both the one and 24 hour contact times, there were two chloroform maxima. The chloroform levels were initially low during the beginning of the growth curve. Maximal chloroform production was reached between days 7 and 10 of growth. After day 10, the chloroform levels dropped and then began to increase again until the second chloroform maximum was reached between days 15 and 21. After the second maximum in chloroform production, the amount of chloroform dropped to low levels toward the later part of the growth curve. The chloroform production from the Pediastrum ECP for both chlorine contact times followed the same general pattern as that of the Pediastrum cells. Selected free chlorine residual data are presented in Tables 4 and 5 for Scenedesmus and Pediastrum. The chlorine doses employed for the ECP and cells were comparable and were generally sufficient to maintain a residual at one hour of chlorine contact time. Tables 4 and 5 show that for both the Scenedesmus and Pediastrum cells and ECP a free chlorine residual was present after one hour of chlorine contact time even during the later portions of the growth curve for all but the lowest chlorine dose (except for the later portion of the Pediastrum ECP growth curve). Therefore, the decline in chloroform levels towards the end of the growth curves for both species' cells and ECP was not due to chlorine limitations.

The leuco crystal violet technique used to determine chlorine levels had a maximum margin of error of ±0.4 mg/L for the 1.5 mg/L dose; ±0.7 mg/L for the 5.0 mg/L dose and ±0.8 mg/L for the 9.9 mg/L and higher doses due to the kinetics of the leuco crystal violet reaction. If the absorbance was read too quickly (before the absorbance plateau was reached) the absorbance was still changing with time. This introduced errors into the chlorine residual calculations. The error factors sited above were calculated on the basis of the maximal error that can be introduced by taking absorbance readings at time zero versus taking the readings after the absorbance plateau had been reached. No statistical analysis of error has been undertaken.

The chemical parameters measured for the experimental controls on all three organisms are given in Tables 6 to 8. Because the terminal THM levels for the references (negative controls) were quite high, in some cases, additional references were prepared in order to check the reagents and solutions employed in the THM experiments to isolate that which was contributing THM precursors to the controls. The references prepared for this purpose and the results of their analyses are given in Table 9. These later references (prepared using chromic acid/sulfuric acid and methanol washed glassware) all had total THM levels of <1.0 µg/L. Glassware handling procedures were critical to elimination of sperious results that appeared because of apparent contamination. Contaminants in the sodium thiosulfate used to quench the chlorine were responsible for the high backgrounds. This could be corrected by purification and gas sparging of the thiosulfate solution.

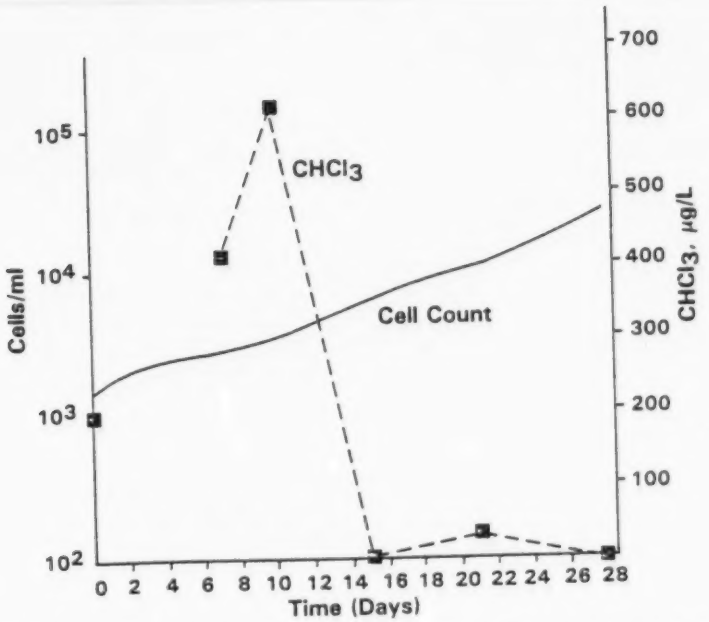


FIGURE 38. Chloroform Production from Pediastrum Cells, 9.9 mg/L Chlorine Dose, 1 Hour Contact Time

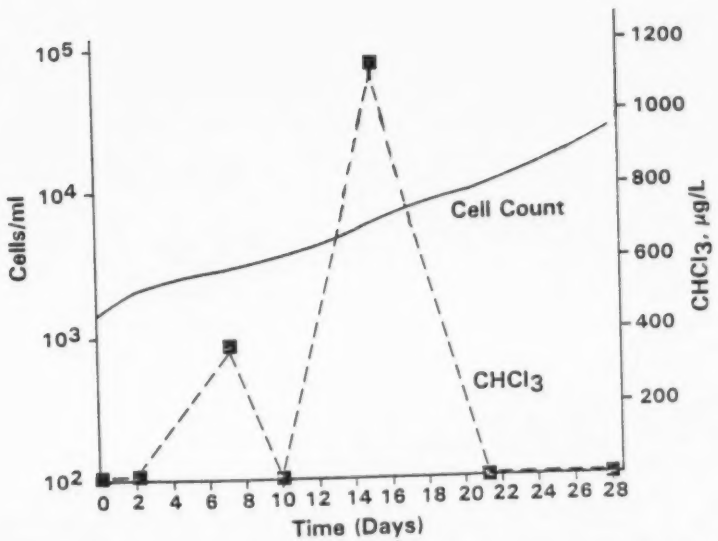


FIGURE 39. Chloroform Production from Pediastrum Cells, 9.9 mg/L Chlorine Dose, 24 Hours Contact Time

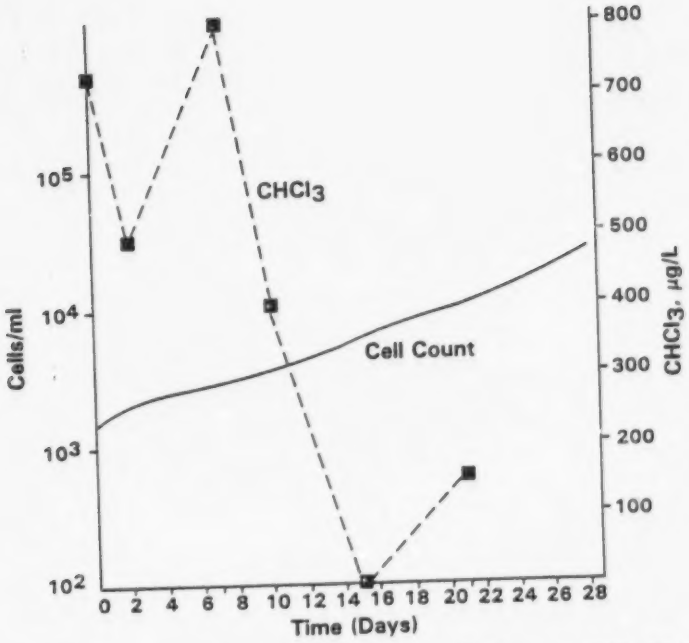


FIGURE 40. Chloroform Production from Pediastrum ECP, 23.7 mg/L chlorine Dose, 1 Hour Contact Time

TABLE 4. Free Chlorine Residual after One Hour Contact Time for *Scenedesmus* Cells and ECP

	Chlorine Dose mg/L	Day of Culture 0	2	7	10	15	21	28
		Free Chlorine Residual, mg/L						
Cells	1.5	1.0	1.0	0.5	0.2	<0.1	<0.1	0.1
	5.0	3.3	2.8	2.6	2.2	1.7	0.7	0.1
	11*	10.8	11.3	10.4	10.9	10.7	9.5	7.7
ECP	23.7	<0.1	<0.1	0.2	1.7	2.9	1.4	2.0
	32.6	0.1	0.1	2.7	4.0	5.8	2.8	4.6

* approximate chlorine dose

TABLE 5. Free Chlorine Residual after One Hour Contact Time for *Pediastrum* Cells and ECP

	Chlorine Dose mg/L	Day of Culture						
		0	2	7	10	15	21	28
		Free Chlorine Residual, mg/L						
Cells	1.5	0.9	1.1	0.9	0.9	0.7	0.4	0.1
	5.0	2.5	2.7	2.5	2.9	2.6	2.1	0.3
	9.9	5.5	5.0	6.0	5.6	6.2	4.4	1.6
ECP	23.7	<0.1	0.1	0.6	0.1	<0.1	<0.1	<0.1
	32.6	0.2	2.5	4.8	1.5	<0.1	<0.1	<0.1

TABLE 6. Chemical Parameter Measurements for Anabaena Cells and ECP Controls

		Chlorine Dose mg/L	Chlorine Residual Free mg/L	Chlorine Residual Total mg/L	TOC mg/L	TKN mg/L	NH_3-N mg/L
WATER	initial	1.5	0.8	1.4	<2	<0.2	<0.01
		5.0	2.7	4.5	<2	<0.2	<0.01
		9.0	5.2	8.1	<2	<0.2	<0.01
	final	1.5	0.8	1.5	<2	<0.2	<0.01
		4.9	2.5	4.1	<2	<0.2	<0.01
		8.7	4.6	8.7	<2	<0.2	<0.01
MEDIUM	initial	6.8	<0.1	1.5	16	1.2	0.04
		23.6	0.1	4.3	15	1.2	0.06
		32.5	*	8.0	15	1.6	0.06
	final	6.8	<0.1	1.8	17	0.60	0.03
		23.7	0.1	3.6	18	0.80	0.07
		32.6	1.3	10.6	15	0.80	0.09

* not analyzed

TABLE 7. Chemical Parameter Measurements for Scenedesmus Cells and ECP Controls

		Chlorine Dose mg/L	Chlorine Residual Free mg/L	Chlorine Residual Total mg/L	TOC mg/L	TKN mg/L	NH_3-N mg/L
WATER	initial	1.5	1.2	2.0	<2	<0.2	<0.01
		5.0	3.3	4.0	<2	<0.2	<0.01
		9.9	5.8	7.6	<2	<0.2	<0.01
	final	1.5	1.2	2.2	<2	<0.2	<0.01
		4.9	3.3	4.3	12	<0.2	<0.01
		9.7	11.7	12.6	2	<0.2	<0.01
MEDIUM	initial	6.8	<0.1	1.8	18	2.8	0.03
		23.7	<0.1	3.6	17	3.2	0.07
		32.6	0.1	9.2	19	3.2	0.08
	final	6.8	<0.1	2.0	17	2.2	0.02
		23.7	<0.1	3.2	18	2.2	0.05
		32.6	0.1	8.8	16	3.8	0.07

TABLE 8. Chemical Parameter Measurements for Pediastrum Cells and ECP Controls

		Chlorine Dose mg/L	Chlorine Residual Free mg/L	Chlorine Residual Total mg/L	TOC mg/L	TKN mg/L	NH_3-N mg/L
WATER	initial	1.5	1.0	1.2	2	<0.2	<0.01
		4.9	2.6	3.5	<2	<0.2	<0.01
		9.9	6.3	11.6	3	<0.2	<0.01
	final	1.5	0.9	1.9	<2	<0.2	<0.01
		5.0	2.8	4.8	<2	<0.2	<0.01
		9.9	5.7	12.1	<2	<0.2	<0.01
MEDIUM	initial	6.8	<0.1	1.8	17	0.6	0.03
		23.7	0.1	3.6	18	0.8	0.07
		32.6	1.3	10.6	15	0.8	0.09
	final	6.8	<0.1	1.3	18	1.8	0.04
		23.7	<0.1	3.2	18	3.0	0.05
		32.6	0.7	10.2	18	3.0	0.06

TABLE 9. Trihalomethane References (Unchlorinated)

Sample	pH	Temperature (°C)	$CHCl_3$ (µg/L)	$CHCl_2Br$ (µg/L)	$CHClBr_2$ (µg/L)
Water	6.88	23.7	<0.1	<0.1	<0.1
Water and Sodium Thiosulfate*	6.88	23.5	0.6-18.6	<0.1	<0.1-0.9
Water and EDTA+ and Sodium Thiosulfate*	9.65	22.7	0.4-18.3	<0.1	2.5-9.9
Bold without EDTA	6.33	22.5	<0.1	<0.1	<0.1
Bold and EDTA+ and Sodium Thiosulfate*	6.74	23.5	<0.1-13.7	<0.1	<0.1

* 1.0 mL of 0.01 N into 10 mL vial

\+ Same concentration as Bold basal medium

SECTION 8

DISCUSSION

The levels of THM produced upon chlorination of either Anabaena cells or the ECP produced by Anabaena varied with chlorine dose and age of the culture. This was true for both the ECP and cells of Pediastrum and Scenedesmus, also. The results support the suggestions by Hoehn et al. (1979) that algae are capable of contributing to the formation of THM upon chlorination. Furthermore the age of culture produced organic compounds that differ in their ability to yield THM upon reaction with chlorine. The present study was not chlorine limited for doses of 23.6 or 32.5 mg/L during the Anabaena growth cycle, yet it was apparent that regardless of the chlorine dose the levels of THM increased as the organism reached the late exponential growth phase and declined as the stationary phase was entered. This general trend for the Anabaena ECP is indicated in Figure 36 and a similar trend for the Anabaena biomass (cells) is shown in Table 3. This observation for Anabaena was further demonstrated by comparing the THM production with the TOC levels during the growth cycle. TOC levels continued to increase with increasing culture age for the Anabaena algal biomass; however, THM levels became stationary or actually decreased after reaching a maximum. The TOC levels observed for the Anabaena ECP were relatively constant over the growth cycle. However, examination of these data show that the slight increase in TOC (days 4-8) corresponded with maximal THM production and, though TOC remained relatively constant from day 10 onward, THM levels fell. The Anabaena algal biomass accounted for higher levels of THM than that observed for the isolated ECP when compared at both the one hour and 24 hour chlorine contact times (Table 10).

On the other hand, the Scenedesmus ECP produced higher chloroform levels after chlorination than did the Scenedesmus cells when compared at both the one and 24 hour chlorine contact times (Table 10). The differences in the chloroform levels produced by the ECP versus the levels produced by the cells were generally greater for the 24 hour chlorine contact time and toward the later part of the growth curve. Subsequent to an initially high chloroform level, which occured just after initiation of the growth curve, the chloroform levels from both the Scenedesmus cells and ECP dropped until approximately day 4 of growth (Figures 35 to 37). At days 4-10 of growth an increase in chloroform production was observed. This increase in chloroform production corresponded with the late exponential/early stationary growth phase.

For both the Scenedesmus cells and ECP, the TOC values generally increased with culture age except for a slight drop in the TOC levels occurring between days 8 and 15 of growth (Figures 28 and 31). The TOC

TABLE 10. Comparison of THM Production from Three Algal Species ECP and Cells

Time in Growth Cycle (Days)	Contact Time (Hrs)	Chlorine Dose	THM, µg/L					
			Anabaena		Pediastrum		Scenedesmus	
			Cells	ECP	Cells	ECP	Cells	ECP
7*	1	Low	315.6	29.6	252.1	944.6	167.4	397.9
	1	High	392.8	45.4	424.0	466.1	<0.1	37.8
	24	Low	408.6	64.6	97.3	120.8	333.1	321.5
	24	Medium	400.3	258.9	343.4	124.7+	359.7	782.8
21	1	Medium	138.0	73.1	481.8	157.1	<0.1	47.3
	1	High	392.8	149.5	31.9	7.1	<0.1	13.9
	24	Low	308.3	57.2	119.6	285.6	<0.1	553.0
	24	Medium	549.2	497.0+	59.7	333.3	<0.1	539.7

* Eight days for Scenedesmus

\+ Only high chlorine dose data available

levels toward the end of the growth curve were increasing for both the Scenedesmus cells and ECP, while the chloroform values declined for the cells (both contact times) and for the one hour ECP. For the 24 hour chlorine contact time the ECP exhibited rather high chloroform levels toward the end of the growth period examined. This may be due to the presence of different organic precursors which require longer contact times to produce chloroform.

The chloroform levels produced by the Pediastrum ECP were comparable to the chloroform levels produced by the corresponding cells (Table 10). The Pediastrum cells and ECP (both chlorine contact times) produced two chloroform maxima after chlorine addition (Figures 38 to 40). The first increase in chloroform levels occurred early in the growth curve with a local maximum of chloroform production occurring between days 7 and 10 of growth. Because the growth curve for the Pediastrum cells was almost linear (relatively constant rate of growth) it was difficult to assign a growth phase to the time periods defined by the days of growth. The entire portion of the growth curve examined for Pediastrum may, however, encompass the late exponential growth phase. The second chloroform level increase for the Pediastrum cells and ECP (both chlorine contact times) occurred between days 15 and 21 of the growth cycle. The time period between the two local maxima of chloroform production (days 10 and 15) showed low levels of chloroform production.

From the levels of THM observed at various times during the growth cycles of the three algae it is possible to calculate a formation potential that relates the amount of THM produced to the number of cells for each species. Table 11 shows this formation potential ($\mu g/10^6$ cells) for the cells and ECP for each algal species at two time points in the growth cycle. The chlorine doses chosen for representation were 23.7 mg/L for the ECP and 5.0 mg/L for the cells. The range of values depends upon the position of the algae in its growth cycle but does not vary greatly between a chlorine contact time of one or 24 hours. Consequently, a bloom of these three species would produce 0.2 to 120 µg THM per 10^6 cells. The ECP produced from these 10^6 cells would produce an additional 0.1 to 50 µg/L of THM. These values are approximate and will vary with growth condition and chlorine dose; however, it is clear that a significant contribution to the overall THM production in a treatment facility can be observed from algal sources.

The TOC data for the Pediastrum cells and ECP (Figures 27 and 30 respectively) indicated that although overall TOC levels tended to increase with culture age, a slight decrease in TOC levels occurred during the time period between days 10 and 15 of growth. The decrease, observed in the TOC levels for the middle portion of the growth curve examined, corresponded fairly well with the observed decrease in chloroform levels during this same time period.

The higher chlorine doses and the longer chlorine contact time (24 hours) for the cells and ECP of both Pediastrum and Scenedesmus produced lower chloroform levels than expected (i.e., compared to the chloroform levels produced by the lower chlorine doses and by the one hour contact time). The reason for this observation has not been established. It is possible, however, that different organohalide compounds were formed rather than haloforms, given

TABLE 11. THM Formation Potential for the Three Algal Species (ECP and Cells) with a Medium Chlorine Dose

Time in Growth Cycle (Days)	Contact Time (Hrs.)	THM Formation Potential $\mu g/10^6$ cells					
		Anabaena		Pediastrum		Scenedesmus	
		Cells	ECP	Cells	ECP	Cells	ECP
7*	24	3.6	2.3	122.6	44.5	21.6	47.1
21	24	0.8	0.7	5.7	31.6	<.001	6.6
21	1	0.2	0.1	45.7	14.9	<.001	0.6

*Eight days for Scenedesmus

higher chlorine doses, a longer chlorine contact time, and different precursor compounds compared to Anabaena. This implies different mechanisms of action.

The TOC level patterns produced by the three algae studied (overall TOC levels increased with culture age for all three species' ECP and cells) seemed to confirm the findings of Hellebust (1974) that cells in lag and stationary growth phases generally release more organic carbon than do growing cells and that autolysis may account for much of the ECP derived from these two phases. Exponentially growing algae release, as ECP, less than 1 to over 50% of their photoassimilated carbon, depending upon species and growth conditions (Hellebust, 1974). The total organic carbon released by Anabaena as ECP includes polysaccharides, simple sugars, glycolate, formate, acetate and lactate. Scenedesmus also releases polysaccharides and simple sugars (Hellebust, 1974).

The TKN and NH_3-N values for the cells of all three algal species were somewhat variable but generally increased with culture age. On the other hand, the extracellular product NH_3-N values for all three species were constant in value with age of culture or dropped toward the later portions of the growth curves. The TKN levels for the ECP of all three algal species generally increased with culture age and then declined slightly toward the later growth stages. The patterns of TKN and NH_3-N production observed from the ECP of Anabaena confirmed the findings of Fogg et al. (1973) that the amount of combined extracellular nitrogen for Anabaena increases during the growth of the cultures. Expressed as a percentage of the total nitrogen fixed, it is highest during the lag and stationary phases of growth and lowest during the exponential growth phase. In general, the relative amounts of extracellular combined nitrogen vary according to environmental conditions and also according to the strain of algae used (Fogg et al., 1973). Hellebust (1974) reported that the largest portion of the extracellular nitrogenous material released by blue-green algae is in the form of polypeptides and only small amounts of free amino acids are found. The extracellular nitrogenous material released by Scenedesmus includes glucosamine, amino acids, and polypeptides (Hellebust, 1974).

The concentrations of $CHBrCl_2$, $CHBr_2Cl$, and $CHBr_3$ for the Pediastrum and Scenedesmus cells and ECP were generally below the detection limits except for several instances in which the bromine-containing volatile concentrations were disparately high when compared to the chloroform levels. Because the concentrations of the bromine-containing volatiles, when present at all, occurred randomly and because they occurred only in a minor portion of the total number of samples, it was deemed likely that the analytical technique for the detection of $CHBrCl_2$, $CHBr_2Cl$, and $CHBr_3$ was observing contaminants rather than the compounds of interest. Accurate figures for the levels of the volatile organochlorine compounds other than chloroform were not obtained with the present analytical procedure. Because bromine was not supplied to the cultures and was not present in the medium constituents except perhaps as very low level impurities (also bromine may be an impurity in the chlorine) it is unlikely that sufficient bromine was present to allow for the formation of high levels of bromine-containing haloforms from the cells and ECP of the algae. If reliable levels of the bromine-containing haloforms were somehow

possible to obtain, it is probable that a growth curve related pattern for these haloform levels would be observed that is similar to the patterns obtained for chloroform. The numbers obtained for the bromine-containing haloforms did not show such a growth curve-related pattern and were indeed apparently random in occurrence. For this reason, it was decided that for Scenedesmus and Pediastrum only the chloroform levels would be used in the data analyses. The technique employed for the Anabaena THM analyses gave no bromoform results and the dibromochloromethane measurements were questionable. For this reason, the total THM data given here for Anabaena actually reflects the concentration levels for chloroform and bromodichloromethane only.

The results presented demonstrate that high concentrations of THM are produced from algal biomass and metabolites. The significance of these results suggest that reduction of THM may be partially accomplished by observation and/or control of the natural phytophankton communities in the water source for domestic water supplies. Clearly, humic and/or fulvic precursors contribute to the THM production upon chlorination; however, this source can only account for a portion of the total THM produced. To date, no complete mass balance has been possible to determine all the precursor molecules that produce THM. This problem is further complicated by the incomplete yields of THM that model compounds produce. It is unlikely that a significant amount of algal biomass can survive through the coagulation and filtration steps of a well operated water treatment plant; however, ECP may persist if they are not destroyed by bacteria or removed in the coagulation process. Furthermore, it is possible for algae to pass through the coagulation and filtration steps if they are present in large numbers or if the treatment plant is not being optimally operated. THM precursors (ECP and cells, as well as humic and fulvic acids) will be present at the point of prechlorination (chlorination prior to coagulation and filtration) employed at many surface water treatment plants. For this reason such materials may allow the levels of THM formed in finished waters to be greater than the THM levels formed if coagulation and filtration is performed prior to chlorination.

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